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RESPONSE OF TWO INBRED STRAINS OF MICE AND THEIR HYBRID TO ANDROGEN[1]

Demonstrated by Mitotic Rate in the Epithelium of the Seminal Vesicles

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(Received February 27, 1956)

Two recent developments in research have dealt with functional differences in the endocrine system among inbred strains of mice. One such development is the strain difference in response to hormone stimulation (Shimkin and Andervont 1941, Trentin 1950, Clarington and Biggers 1955). The other is the so-called "hormonal imbalance" which has been considered an etiological factor in some types of cancer. Such imbalance may exist in certain inbred strains of mice (Woolley, Fekete and Little 1939, Bittner and Huseby 1946, Bittner 1950, 1952). Perhaps basic to both are the differences in sensitivity and level of hormone secretion which are in turn primarily dependent upon the genetic backgrounds of the different mouse populations.

In a previous study (Chai, 1956) two different inbred strains of mice and their F_1 hybrids were treated with testosterone propionate. One purpose was to gather information concerning the pattern of strain differences in response to this hormone, and to evaluate the relative levels of testicular androgen secretion. Significant differences in response as measured by seminal vesicle size were found between the two inbred strains of mice studied and the values of the F_1 hybrids fell between those of the parents.

In the present study the same genetic kinds of mice and the same hormone treatment were used, but in addition colchicine was given as an agent for arresting mitoses (Fleischmann 1939, Tislowitz 1939, Gaulden and Carlson 1951). The proportion of cells in mitosis in the epithelium of the seminal vesicles was taken as the index of response.

[1] This investigation was supported by a research grant C-1074(C5) from the National Cancer Institute of the National Institutes of Health, Public Health Service.

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Thus the present investigation was designed to examine the difference between these genetically different mouse populations in response of the seminal vesicles at the cytological level. The results may contribute concomitantly to the understanding of the phenomenon of organ growth *in vivo* in relation to androgen stimulation.

Materials and Methods

The BALB/c and A/Jax inbred mouse strains and their F_1 hybrid (CAF_1) produced by mating BALB/c female and A/Jax male were chosen for the experiment. Only males weighing between 9 and 12 grams at the time of weaning (approximately 30 days old) were used. There was a total of 210 mice used in this experiment.

The mice were bilaterally castrated on either the day of weaning or the following day. It was felt that at this age the animals were growing rapidly and would be highly responsive to the experimental treatment; also that their own testicular androgen secretion would be presumably at a lower rate and would be depleted within a short time following castration. On the seventh day after castration, the mice, in 5 groups of ten, were started on their hormone regimen. Testosterone propionate made up as a suspension in distilled water was serially diluted to five different concentrations (.256, .128, .064, .032 and .008 mg per tenth of a cc). Three subcutaneous injections of 0.1 cc were given at a twenty-four hour interval. Twenty-four hours after the last hormone administration, the mice were given an injection of .05 mg colchicine in 0.1 cc distilled water. In addition to the hormone treated animals, 10 castrated control and 10 intact control animals received the colchicine treatment but no hormone.

All mice were sacrificed eight hours after the administration of colchicine. The seminal vesicles were removed and the coagulating glands adherent to them were detached. One seminal vesicle from each mouse was fixed in Vandergrift's fixative, embedded in paraffin, sectioned longitudinally at five micra and stained with Meyer's hematoxylin and eosin. Ten sections at regular intervals along the rows of sections were examined under oil immersion. One field was randomly selected from each of the ten sections for the examination. The number of epithelial cells per .060 mm^2 were counted with the aid of a graded eye piece. Mitotic cells in the late prophase, metaphase and early anaphase were counted separately and added to all their epi-

thelial cells to give the total number. The mitotic index (mi) was computed according to the following formula:

$$\text{mi} = \frac{\text{total number of mitotic cells/field}}{\text{total number of epithelial cells/field}}$$

The mean value of ten fields was taken as an unbiased estimate of mitotic index for each mouse and was used in the analysis. The total number of epithelial cells counted per field was in most cases more than one thousand. The mitotic cells were, in practically all cases, easily distinguished from both healthy cells in other phases and from cells undergoing degenerative changes.

Results and Analysis

The means and standard deviations of the mitotic indices for each dosage group within each genotype were computed (Table 1). According to these values the A/Jax strain ranked the highest, CAF_1 hybrid next and the BALB/c strain the lowest. Within each genotype the mean mitotic index for the intact control group was lower than that of the treated group. The relative order of magnitude for the three control groups was the same as the treated, that is mi(A/Jax) > mi (CAF_1) > mi (BALB/c). No mitosis was found in the castrate control mice.

An analysis of variance was performed to test the differences in response among the three different genotypes. Since angle transformation is generally recommended for values in percentages in the analysis of variance, the mitotic index obtained for each mouse was transformed according to the equation, angle = arc sine $\sqrt{\text{mitotic index}}$. The analysis was then continued following the method illustrated by Snedecor (1949). The variance attributed to genotype was significant ($p < .01$) (Table 2). Testing the difference of the means of any two groups, only the difference between A/Jax and BALB/c was highly significant; there was no significant difference between CAF_1 and either parental strain. The genetic differences contributing to the total variation was estimated to be 43%.

Comparisons of the variation of response between the inbred and hybrid mice within dosage and within genotype on the angle basis were made (Table 1). The average variance of the CAF_1 fell between that of the parental strains and the differences were not significant.

Because the standard deviations on the actual scale appeared to be dependent upon the means, the angle transformation may be suitable in this case for correcting the dependency (Finney 1952).

TABLE 1

The Means and Variances of Mitotic Index (mi) and Rate of Cell Growth (mi/T) Under Different Treatment Within Each Genotypic Group of Mice.

		Castrate control	Castrate + Hormone					Intact control
Testosterone propionate		0	.008	.032	.064	.128	.256	Average	0
BALB/c	mi	0	1.1	2.5	3.3	3.1	6.1	3.22	1.0
	s^2	0	.28	.46	.67	.49	2.89	.96	.61
	s^2 angle	0	3.80	2.10	1.90	1.51	3.81	2.62	3.43
	mi/T	0	.14	.31	.41	.39	.76	.40	.13
CAF_1	mi	0	2.0	3.8	4.0	5.8	6.8	4.48	1.6
	s^2	0	.81	1.39	1.15	.88	3.24	1.49	2.25
	s^2 angle	0	3.30	3.39	1.48	.80	6.03	3.00	6.64
	mi/T	0	.25	.48	.50	.73	.85	.56	.20
A/Jax	mi	0	2.6	4.1	5.9	8.9	9.4	6.18	2.1
	s^2	0	1.96	4.84	2.56	1.59	6.76	3.54	1.44
	s^2 angle	0	6.04	7.15	3.93	2.19	5.51	4.96	5.76
	mi/T	0	.33	.51	.74	1.11	1.18	.77	.26

The coefficients of correlation and regression of mitotic index on dosage of hormone in each genotypic group was computed.

Genotype	*Correlation coefficient*	*Regression coefficient*
A/Jax	.76	$6.41 \pm .79$
CAF_1	.82	$4.72 \pm .47$
BALB/c	.82	$4.92 \pm .49$

Both the correlation and regression coefficients are significantly different from zero but not from each other. Although the regression coefficient in A/Jax appeared higher than the others the differences between any two is not significant. A graphical illustration of the dose-response relationship is given in Fig. 1.

Included also in Table 2, the average rate of cell growth per hour ($\lambda = mi/T$) was obtained for each dosage group. This value is useful for the purpose of comparison with the growth rates of other tissue or of tumor cells. Since the effects of colchicine, in arresting mitosis, take place immediately upon its injection and since it was administered 8 hours before autopsy, the T value in hours in this case is 8.

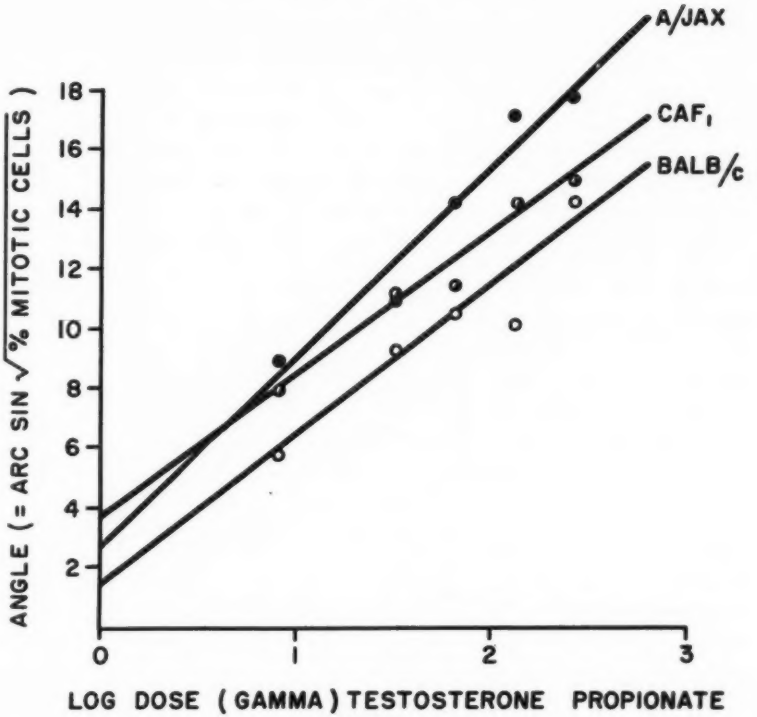

FIGURE 1

Illustrating the linear regression of response of mitotic rate on graded doses of testosterone propionate in each genotypic group of mice. The regression lines were constructed according to the following equations: A/Jax, $\hat{Y} = 2.63 + 6.41X$; CAF_1, $\hat{Y} = 3.71 + 4.72X$; BALB/c, $\hat{Y} = 1.30 + 4.96X$; where X is log dose and $\hat{Y}$ is the predicted response on angle basis.

TABLE 2

Analysis of Variance of Mitotic Index Among Different Genotypic Groups of Mice.

Source of variation	Degree of freedom	Mean squares	F test	Variance
Genotypes	2	185.53	$p < .01$	3.39
Dosages	4	290.60	$p < .01$	
G × D	8	12.53	$p < .01$	
Within dosage and genotype	135	4.61		4.61

Percentage of variance attributed to genetic difference $= \frac{3.39}{3.39 + 4.61} = 42.4$

Discussion

The significant differences in mitotic indices among the three different groups of mice is not surprising, but the order of magnitude of response deserves scrutiny. As mentioned earlier, in a previous experiment (Chai, 1956) by taking the weight of the seminal vesicles as an index of response, a rank order reverse to that found here was obtained; that is, BALB/c mice gave the highest response, CAF_1 mice the next and A/Jax mice gave the least. It is known that organ growth is often found to obey the exponential law, $N = N_o e^{\lambda_n T}$ and $V = V_o e^{\lambda_v T}$, where N and N_o = the final and initial number of cells, V and V_o the final and initial cell volume and the rate of change in time T (Hoffman 1953). With organs of the same size those which have higher rates of cell multiplication would require lower rates of increase in cell volume in order to reach this size and vice versa. According to the results of the previous and the present experiments, it seems reasonable to conclude that BALB/c mice had larger sized but fewer cells in the epithelium of the seminal vesicles than A/Jax mice. The total contribution of the additional cells to the organ size in A/Jax, however, did not counter balance the deficiency due to smaller cell volume sufficiently to make their average seminal vesicle weight comparable to that of BALB/c mice.

Cavazos and Melampy (1954) have found that androgen causes cell volume increase in the epithelium of the seminal vesicles in rats. Although no direct measurement of cell volume was undertaken in the present investigation, there appeared to be a great increase of cell volume in the hormone treated mice.

Previous investigations (cited by Hoffman 1953) show that the growth rate (mi/T) of cells of mouse ascites tumor was 2.5/hr., of DBr mouse adenocarcinoma 2.7/hr. and of transplantable rat hepatoma .60/hr. The growth rate of cells in the seminal vesicles of the intact control mice was lower than that recorded for mouse tumor cells, but in those subgroups which were treated with higher dosages of hormone, the rate of growth was higher than that of the transplantable hepatoma in rats. The higher growth (mi/T) rate of the epithelial cells in the seminal vesicles of A/Jax mice may indicate a higher sensitivity to hormones of the somatic cells in general in this strain. Chances for abnormal growth to occur are thus more favorable in case of hormonal imbalance. This is perhaps a bit too speculative and

needs more experimental evidence. Nevertheless, the present results showed differences of the basic pattern of growth response of the seminal vesicles between the two inbred strains of mice, which reflects diverse metabolic patterns.

Tislowistz (1939) found that mitosis in the epithelium of the seminal vesicles began at 27 to 31 hours after a single dose of injection of testosterone propionate in mice from his laboratory. It is possible that the latent period of mitotic activity in response to androgen and colchicine treatment might be different among the genetically different groups of mice. In the present experiment, however, three doses of hormone were administered at 24 hours of interval. It is known that testosterone propionate has considerable prolonged effect in the animal body. Each injected dose of hormone would have some carried over effect at the time when the next dose was given. Thus the mitotic activity in the epithelium of the seminal vesicles had been taken place far ahead the time when the colchicine was injected which was 72 hours after the first dose of hormone injection. We further expect that each animal of either genotype the mitotic activity caused by the hormone stimulation had remained at a fairly constant rate during the time when the colchicine was effective. In other words, we felt that this designed treatment should be able to minimize the genetic differences of mice in reactivity with respect to the time of hormone being given, if any.

The colchicine treatment was given at a dosage just below the toxic limit. Such a high dosage, in comparison with lower dosages, should reduce the genetic differences in sensitivity to colchicine with respect to time. Yet, we did not know whether these differences, if existed, have been removed out entirely. It may be safe to say that as a possible source of contribution to the genetic differences in sensitivity, this factor should not be disregarded without factual proof.

Hooker (1939) showed in rats that the period from 40 days (beginning puberty) to 60 days old was most sensitive to androgen stimulation. The mice used in the current experiment were about 37 days old at the time of starting the hormone treatment, an age just after puberty for mice of those strains and hybrids. Although no experimental proof was obtained in the present study, in accord with Hooker on rats, it is believed that those mice should be in the most sensitive stage when the treatment was applied. It is obvious that in the study of this sort there many factors, which may be com-

pounded to the real genetic differences in response to androgen stimulation, could be involved. We tried to keep them as constant as possible so that comparison of mitotic activity in response to the given treatment can be made on a logical basis.

The approximate intermediacy in response of the hybrid mice in relation to the responses of the parental strains (although a slight tendency toward the BALB/c in lower doses) is a genetic consequence often observed. Similar results have been obtained using growth and many other traits as a criterion. As far as responses to different hormones are concerned, in investigations where estrogen was administered to different inbred mice and their F_1 hybrids, some showed that the F_1 hybrid gave a response between that of the parental strains (Shimkin and Andervont 1941, Trentin 1950) while others showed the response of the F_1 hybrid to be beyond that of the parent giving the higher response (Clarington and Biggers 1955). Feeding thiouracil to chickens, Andrews *et al.* (1953) showed that the gross thyroid weights of the crosses were nearly midway between the gross gland weights of the parental inbred lines but that the relative thyroid weights of the crosses were similar to those of the parent with lower relative thyroid weight. These findings seem to suggest that the pattern and/or intensity of response is dependent upon the genetic constitution of the test animal and the nature of the substance applied.

Although the estimated genetic variance contributed a considerable amount (43%) to the total variance, the genetic basis underlying the response must be very complicated due to interaction of endocrine systems. These systems are very likely under control of many genes with similar and/or different effects, making their study in genetic terms very difficult. Nevertheless, since the hormones play a very vital role in the body of an organism, genetic study in this field is sorely needed.

The insignificant differences in regression coefficients of mitotic indices on hormone dosages among the three different groups of mice was expected because mitotic index was calculated on the percentage basis. Should an absolute count of the mitotic cells be undertaken, the regression value in the A/Jax mice would be much higher due to the geometric progression of cell multiplication. Both the significance of regression coefficient within the genotype, and the non-occurrence of mitosis in the castrate control mice indicate the unique property of androgen in influencing cell growth of the seminal vesicles. It seems

that the application of still higher doses of androgen might be worthwhile in order to find out the limit of the rate of mitosis due to stimulation by this hormone. At the same time qualitative observations of the mitotic cells could be made.

Theoretically since the F_1 hybrids have a superior buffering property to that of the homozygous individuals, a smaller variation would be expected (Lerner 1953). This was apparently not so in the present case. The magnitude of variances of the mitotic indices on the transformed scale for the three different genotypic groups was quite similar; the variance in the F_1 hybrid was slightly smaller than that in the A/Jax but was larger than that in the BALB/c. No reasonable interpretation can be made.

Summary

A quantitative study of the response of the seminal vesicles to testosterone propionate, as indicated by the mitosis of cells in the epithelium was carried out for two inbred strains of mice (BALB/c and A/Jax) and their F_1 hybrid (CAF_1). The value of the mitotic index of the epithelial cells of the seminal vesicles in A/Jax mice was shown to be significantly greater than that of BALB/c mice, while that of the CAF_1 fell between values of the parental strains. On an angle-transformed scale, the rise of the mitotic index with respect to the increase of dosage of testosterone propionate was found to be linear within the range of the dosage applied. The variance of mitotic indices within each genotypic group were similar in magnitude; the F_1 hybrid showed a slightly smaller value than that of A/Jax but larger than that of BALB/c.

Possible interpretations and inferences are discussed.

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A COMPARISON OF GENETIC AND ENVIRONMENTAL PRINCIPAL COMPONENTS OF MORPHOGENESIS IN MICE

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Students of growth have become increasingly aware of the importance of the interrelationships among the body parts to the understanding of the mechanisms of morphogenesis. This is evidenced by the evolution of the methods used in the analyses of growth and form. Perhaps the most stimulating innovation in the field of biometrical morphogenetic studies was that of the Thompsonian coordinates (D'Arcy Thompson 1917, 1942). Although it is hampered by lack of a means for mathematical analysis, it illustrates well the integration of the parts into a whole, meaningful organism. The elucidation of the methods of allometry by Huxley (1932) brought a method capable of mathematical analyses into prominent use. It has become evident, however, that even this method is quite limited, for it deals with but two parts at a time and in most cases it is used only as a descriptive tool. More effective means for studying relative growth have been adapted to special cases (e.g. Medawar, 1945; or Richards and Kavanaugh, 1945).

Lately, the method of factor analysis, a different and very promising means of analyzing morphology, has come into use from the field of psychology (see Thurstone, 1947). Although this method is ordinarily referred to as "factor analysis" by the psychologists, in this paper it will be referred to as "principal component analysis" in order to avoid confusion between the geneticists' and the psychologists' connotation of the term "factor." This method has many advantages over those mentioned above. First, many morphological measurements can, and for greatest efficiency should, be analyzed at the same time; second, it can be easily adapted to standard methods

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of quantitative genetics for more elaborate analysis of the genetics of morphology; and third, it adapts itself exceedingly well to the principles pertaining to the Thompsonian coordinates.

The extraction of principal components is a method whereby many variables, which would almost invariably possess a great deal of redundancy, are reduced to a set of uncorrelated variables (the principal components), thus ridding the data of its redundancy. The method has advantages over other methods of its kind in extracting, in descending order, the components which account for the greatest amount of variance. The original variables, as will be seen below, can be defined in terms of these principal components.

Several psychologists (Burt 1943, Burt and Banks 1947, and Thurstone 1947) have made use of principal components or related methods in analyzing morphological data on humans. Wright (1932 and 1954) and Howells (1953) have promoted to some extent the use of these methods in genetic studies. However, further synthesis of these methods with those of quantitative genetics may prove to be highly useful.

The separation of the genetic and environmental portion of variance and covariance prior to extraction of the principal components is of primary concern for ultimate analyses and utilization of the components. Since there is no evidence available indicating the two sources of control would possess similar components, their (statistical) separation is necessary in order to avoid analyzing compounded effects which would have no obvious biological meaning. The purpose of the present study is to make such a separation, thereby providing a basis for comparing the genetic and environmental components, and for emphasizing the need of such a separation in other studies. In addition, this separation is a necessary step in the utilization of the method in quantitative genetic studies of morphogenesis. The results may be examined with these ends in mind.

Materials and Methods

The basis of the subsequent statistical separation of the genetic and environmental portions of the variance (and covariance) lies in the theoretical expectation that all genetic variance lies between highly inbred strains and none within strains (Wright 1921). For this reason mice from as many different inbred strains as practicable were obtained for making the subsequently described skeletal measurements.

Those strains used in the axis data have been described (Bailey, 1956); those used in the mandible data are shown in Table 1. In this study we are assuming that the environmental genetic interaction is negligible. However, one should always be aware that this source of influence may be of unrealized importance.

TABLE 1
Inbred Strains Used in the Mandible Study and Their Inbreeding Histories.

Strain	Known Generations Brother-Sister Inbred	Strain	Known Generations Brother-Sister Inbred
129	17+	C57BL/10	50
Balb/Jax	93	C57Br/a	90
C57BL/6	44	RIII/Jax	20
LP	46	DBA/2Emv	18+
MA/Jax	33	C57BL/4p	46
CBA	88	C3H/Ks	17+
Balb/cAn	83	C57L/Jax	61
C57Br/cd	82	HA/Jax	23
P/Jax	55	C3H/Jax	35
DBA/1	18+	AKE	19
A/Jax	92	C57BL/^{at}	19
$C3H_{eb}$	15	CE	13

The data on the axis and mandible were taken from independent collections of skeletons, prepared by the papain-digestion technique. The axis data, obtained from the earlier study, utilized adult male skeletons of 18 inbred strains with three litters per strain and two individuals per litter. The mandible data, obtained solely for inclusion in this study, utilized both male and female skeletons from 24 inbred strains with two litters per strain and 1 male and 1 female per litter.

The axis data have been described in detail (Bailey, 1956). They consisted of measurements shown in Figure 1 as represented by:

AH—arch height
AW—arch width
L—lamina length
P—pedicle length
T—transverse process length
B—base length
CH—centrum height
CW—centrum width
I—innominate bone length (not shown)

The innominate bone length was included in the previous study as a standard for general skeletal size.

The mandible measurements are shown in Figure 2. All of these measurements originate from the posterior margin of the third molar alveolus of the (left) mandible and extend to each of the processes or incisurae:

A—to anterior margin of first molar alveolus
B—to anterior margin of incisivus alveolus
C—to processus angularis
D—to incisura mandibulae ventralis
E—to processus condyloideus (ventral capitular border)
F—to processus condyloideus (dorsal capitular border)
G—to processus coronoideus

Analyses of variance and covariance were utilized in both mandible and axis sets of data so that both inter-strain (genetic) and inter-litter, intra-strain (environmental) variances and covariances could be estimated. From these statistical estimates, product-moment correlation coefficients of two types, genetic and environmental, were computed for all combinations of measurements within each (mandible or axis) set. Thus for each of the two bones, two sets, or matrices, of

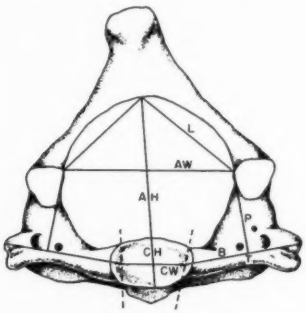

FIGURE 1
Drawing of posterior view of axis showing dimensions used.

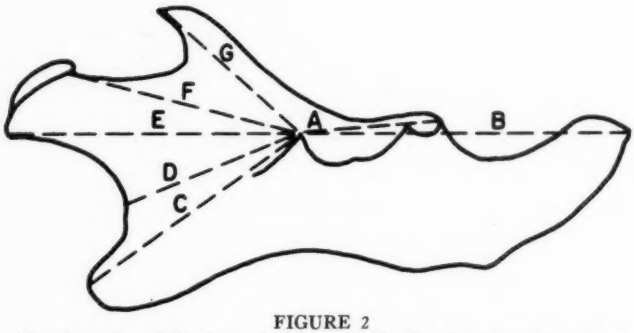

FIGURE 2
Drawing of medial view of left mandible showing dimensions used.

correlation coefficients were derived, one genetic and the other environmental. From these correlation matrices, the principal components have been extracted.

A simple method of extracting principal components as developed by Hotelling is outlined by Fruchter (1954). Related methods have been more extensively used by psychologists. However, the extraction of principal components is more useful in the utilization proposed here. Since the present study is aimed primarily at illustrating the need of analyzing the genetic and environmental components separately and since the genetic correlations do not possess many degrees of freedom, only the first two principal components have been extracted from each correlation matrix. Unities have been used in the diagonals.

Results

The correlation matrices derived from the mandible data are given in Table 2 in a somewhat condensed form. Those derived from the axis data have been given (Bailey, 1956) and need not be reported here.

TABLE 2

Correlation Coefficients of Mandible Measurements. Environmental Correlations Are in Upper Right Half of Table; Genetic Correlations Are in the Lower Left Half.

	A	B	C	D	E	F	G
A	1.000	0.707	—0.675	—0.781	—0.591	—0.319	—0.164
B	0.813	1.000	—0.425	—0.600	—0.192	0.047	0.067
C	—0.003	0.402	1.000	0.889	0.847	0.563	0.238
D	—0.079	0.396	0.770	1.000	0.726	0.324	0.123
E	0.458	0.690	0.519	0.684	1.000	0.657	0.388
F	0.245	0.465	0.400	0.531	0.879	1.000	0.510
G	0.468	0.559	0.387	0.238	0.611	0.545	1.000

The principal components extracted from each correlation matrix are presented in Tables 3 and 4. The contribution which each component makes to the total variance is shown in the bottom row of each table. It is seen that the genetic and environmental components remove close to 80% of their respective variances for the mandible measurements, whereas they remove around 60% for the axis measurements.

Vectorial representations of the measurements, based on the principal components obtained, are shown in Figures 3 and 4. In these figures one can more easily see differences and similarities in the genetic and environmental configurations.

Axis. Both genetic and environmental components appear to have distinguished between the neural arch measurements and the centrum (and innominate) measurements. (The measurements P, pedicle length, and B, base length, are exceptional since they are compound measurements, being dependent upon other dimensions of the arch

TABLE 3
Principal Components of the Axis.

	Genetic		Environmental	
	I	II	I	II
AH	.02	.63	—.23	.67
AW	—.61	.51	.52	.46
L	—.49	.71	.49	.64
P	.71	—.16	.22	.22
T	—.10	.58	—.35	.74
B	—.90	.11	—.76	.39
CH	.93	.30	.63	.24
CW	.46	.57	.85	—.12
I	.60	.63	.76	.07
% Variance	37.7	26.0	33.5	21.0

TABLE 4
Principal Components of the Mandible.

	Genetic		Environmental	
	I	II	I	II
A	.54	.79	—.84	.33
B	.82	.41	—.55	.69
C	.66	—.54	.93	.02
D	.69	—.63	.91	—.27
E	.94	—.05	.87	.29
F	.81	—.13	.61	.64
G	.73	.25	.37	.67
% Variance	56.4	22.3	56.8	22.8

and centrum, see Figure 1.) A non-mathematical rotation of the axes (components) can be made (see Thurstone lit. cit.), by approximation based on interpretation of components discussed below, to the position of the dashed line in each case. It then can be seen when comparing the genetic and environmental spaces that there is a reversal of order of the arch dimensions about the arch component and a reversal of order of the centrum and innominate dimensions about the centrum component.

Mandible. The mandible measurements arrange themselves in similar ways in both the genetic and environmental spaces. However, the measurements are spread out to a much greater extent in the

environmental space. A non-mathematical rotation of the axes by approximation (as for the axis) in both spaces has been indicated by the dashed lines.

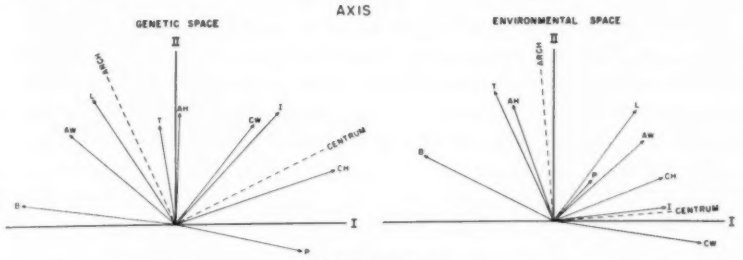

FIGURE 3
Vectorial representation of principal-component matrices of axis.

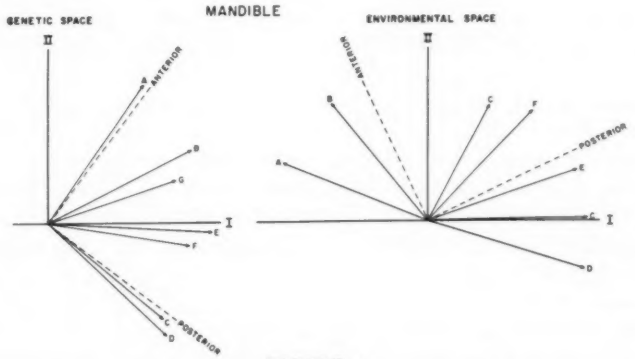

FIGURE 4
Vectorial representation of principal-component matrices of mandible.

Discussion and Conclusions

Developmental Interpretations of Components. Just as the psychologists look for behavioral interpretations for their "factors," we may look for anatomical interpretations of the components derived here. The morphogeneticist has a great advantage in such interpretations for he can draw information from embryological, functional, and spatial relationships. These are denied the psychologist. From the two principal components of the axis for both the environment and genetic sources, one may conclude that the components most likely

represent "arch" and "centrum" sources of control. Such a conclusion is supported by the findings of the embryologists (see Holtzer 1952), where the independence of these two portions are observed in development.

The developmental components of the mandible are more difficult to define and for this reason are termed merely "anterior" and "posterior" components. The anterior component seems to be more closely concerned with the variations of the teeth while the posterior component seems to be concerned with variations of the muscle-attachment and articulation processes which might be further interpreted as a "muscle-advantage" or "leverage" component. Other interpretations could be hypothesized, however.

The amounts of variance accounted for by the components indicate that a greater number of components may be required to define axis measurements than mandible measurements. This may be taken to indicate that the developmental processes of the axis are of a greater complexity than those of the mandible. This observation, on the other hand, may be the result of differences in the kinds of dimensions used on the two bones.

Similarities of the genetic and environmental spaces. Similarities between the arrangements of the measurements are found in the genetic and environmental spaces for both the axis and mandible measurements. This indicates that both genetic and environmental factors are affecting the same pathways—although no doubt with emphases of affects distributed somewhat differently. This same interpretation was made in the earlier study of the axis data. If such a close relationship between the environment and the genome is truly in effect, the following consequences may be inferred. First, the increase of the genotypic range by an increased range of environmental stimuli is possible and has been discussed (Bailey, 1956). Second, the intimacy between gene action and environmental affect may have experimental advantages not realized before. The correction of hereditary deficiencies and anomalies by environmental treatments during development is given more promise, and treatments of a more subtle nature, for example, than those used by Runner (1954), are implied.

Differences of the genetic and environmental spaces. The reversal of the arch measurements and the centrum and innominate measurements about their rotated axes is not readily explained. At the moment the most suitable explanation is that a system remotely affected

by an environmental stimulus will tend to compensate for the effect. Thus, the "arch" measurements tend to compensate for any indirect environmental effects they receive by way of the centrum component and vice versa. This difference is not seen in the mandible spaces, perhaps because the measurements and the bone are of a simpler nature. On the other hand, the difference of the two spaces of the axis may be due more to insufficient extraction of components. If a greater number had been extracted, perhaps more similar configurations in the genetic and environmental hyperspaces would have resulted. More extensive data than used here are required to resolve this point.

A different effect is seen in the mandible environmental space when compared to that of its genetic counterpart. There is a decided dispersal of the measurements through the space. This may be explained by the fact that all measurements were made from a common point. Variation of this point (see Figure 2) in relation to the peripheral points of measurement, would tend to create negative correlations between the measurements. Thus the original positive correlations would decrease while their angular distances in the space would increase.

One purpose of the present separation of the variance into genetic and environmental portions and the subsequent extraction of principal components has been to emphasize the need of such separations. In the present case the components were somewhat similar, although differences were evident. In other cases the components may not be so similar and the biological meaning of the components when not statistically separated may not be so clear due to the compounding of the environmental and genetic effects.

Predictive value of the genetic space. The genetic and environmental spaces of Figure 3 contain in a much condensed form the same information obtained by the "simulated artificial selection" procedure done in the previous study on the axis (Bailey, 1956). By the previous procedure, the increase of one dimension, arbitrarily chosen, would bring about concomitant changes (due to genetic correlation) of other dimensions and the consequence to the shape of the axis could be seen. The same information can readily be obtained from Figure 3. A change in any of the measurements would result in a concomitant change in the others to a degree depending upon their proximity to the originally altered dimension. The figures of the al-

tered vertebral shapes in the previous study agree remarkably well with predictions of vertebral shape changes made from Figure 3.

The foregoing paragraph suggests the use of the genetic space as a means of predicting the effects of artificial or natural selection on morphology. If a multidimensional genetic space were established for a population for morphological (or physiological) traits, it seems probable that an analytically more effective means of predicting the final outcome of selection could be made than are at present available. Such an application of the method is now under investigation by the writer.

Summary

Many dimensions of the axis and mandible have been measured on mice from many inbred strains. Through the analyses of variance and covariance of these data for the axis and mandible separately, both environmental and genetic correlation matrices have been derived.

The first two principal components have been extracted from each of the four resulting correlation matrices. The corresponding genetic and environmental components were then compared.

The first two components of the axis appear to be interpretable as "arch" and "centrum" components for both environmental and genetic sources of control.

The first two components of the mandible are interpretable as "anterior" and "posterior" components. The anterior component deals mainly with the tooth measurements while the posterior component deals mainly with the muscle attachment processes or "leverage" measurements.

The arrangements of the measurements in the genetic and environmental spaces (vectorial representations) are very similar. This has been interpreted as meaning that the genetic and environmental arrays of stimuli operate through quite similar developmental pathways. Differences in these arrangements, however, were evident and suggestions as to their probable causes were given.

The need of a separation of the environmental from the genetic sources of variance has been emphasized in the discussion.

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THE EFFECT OF CORTISONE ON SOME CHEMICAL CONSTITUENTS OF DEVELOPING CHICK LIVER[1]

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The purpose of this investigation was to study the effect of cortisone on growth and certain chemical constituents of the liver of developing chicks. Karnofsky *et al.* (16) found that cortisone causes a marked decrease in over-all growth of chick embryos, and Evans (10) reported that the liver in cortisone-treated chicks is disproportionately reduced in size. Hence, the chick liver appears to be an ideal organ for a more detailed study of the effects of cortisone.

Many investigators have reported that cortisone inhibits the synthesis of ribosenucleic acid and/or desoxyribosenucleic acid (2, 6, 9, 17, 26, 30, 31) and it has been suggested that the decrease in growth resulting from administration of cortisone is due to an inhibition of nucleic acid synthesis with a resultant decrease in mitosis (9). The possibility also exists that cortisone causes a block in some mechanism other than nucleic acid synthesis. The relationship of hormones to mitotic activity has recently been reviewed by Bullough (5) who suggests that the glucocorticoid hormones may influence mitotic activity by affecting the energy requirements of the cell, especially carbohydrate transformations.

In the present investigation the content of nucleic acids, protein nitrogen, total nitrogen and glycogen were evaluated on the basis of total amounts per liver, per cell and per unit weight of liver. The results indicate that the decrease in liver size in fetal chicks treated with cortisone cannot be adequately explained on the basis of a decreased synthesis of nucleic acids or protein.

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Material and Methods

New Hampshire Red eggs were incubated in a forced draft incubator at a temperature of 37.5°C. and a relative humidity of 84-88%. Under these conditions there was little variation in embryonic material. A total of 600 eggs was used in eight different series containing an average of 75 eggs each. Cortisone-treated embryos received 1.0 mgm. of cortisone acetate (Merck) suspended in 0.3 ml. of normal saline while control embryos received 0.3 ml. of normal saline. The injections were made onto the chorio-allantoic membrane after eight days of incubation.

At daily intervals from nine through nineteen days of incubation the embryos were removed from the eggs and immediately weighed to the nearest tenth of a gram. The entire liver of each embryo was rapidly dissected out and weighed to the nearest milligram on a Roller-Smith torsion balance. From nine through fourteen days of incubation it was necessary to pool the livers from several embryos in order to obtain sufficient material for analysis. The livers were treated as follows.

Cell Counts: A carefully weighed portion of each liver was used for a cell count. These portions were taken alternately from the right and left lobes in both the pooled and single samples in order to eliminate any possible heterogeneity between the lobes. The samples were diluted 1:20 with 0.25 M sucrose-0.0018 M $CaCl_2$ (14) and homogenized with a plastic pestle in a glass homogenizer immersed in an ice bath. A 0.5 ml. aliquot of the homogenate was then added to 4.5 ml. of 0.25 M sucrose-0.0018 M $CaCl_2$ and stored in the ice bath for a period of 30 minutes. The nuclei were then stained by adding 5.0 ml. of a solution containing 80 mg. of crystal violet in 100 ml. of 3% acetic acid. The sample was then shaken on a constant speed shaker for a period of 30 minutes. Nuclei from the sample were counted using a standard hemacytometer. Two separate counts of nuclei in five large squares were taken from each sample following approved laboratory technique (20). The average of these two counts was expressed as the number of cells per milligram wet weight of tissue. The values for the chemical constituents per cell were derived by dividing the amount of each constituent per milligram wet weight of tissue by the number of cells calculated per milligram wet weight of tissue.

Determination of nucleic acids: The remaining portion of the liver was carefully weighed and immediately frozen in a mixture of alcohol and dry ice. The frozen portions were stored in a deep freeze unit at a temperature of —16°C. The frozen samples were homogenized in either nine or nineteen volumes of distilled water in an all-glass homogenizer immersed in an ice bath. Nucleic acids were extracted from an aliquot of 1.0 or 2.0 ml., depending upon the size of the sample, according to the procedure of Schneider (33). The residue remaining after the extraction of nucleic acids was dissolved in 5.0 ml. of 1 N KOH and used for the determination of protein nitrogen. The amount of nucleic acid in the isolated fractions was determined using a Bausch & Lomb Spectronic 20 spectrophotometer. Ribosenucleic acid (RNA) was measured with the Mejbaum (27) modification of the Bial orcinol test for pentose; desoxyribosenucleic acid (DNA) was measured with the Dische (8) diphenylamine test for desoxypentose.

Standards: Standard solutions of DNA and RNA were prepared from material purchased from Nutritional Biochemical Corporation. Carefully weighed portions were dried to constant weight in a dessicator in order to determine the per cent dry weight of the standard. Phosphorus determinations were done on each of the standards following the procedure of Fiske and Subbarow (12). The standard curve was then corrected for water and phosphorus content of the standards, and experimental results were expressed as RNA phosphorus (RNAP) and DNA phosphorus (DNAP).

Recovery experiments: Known amounts of the dry RNA and DNA standards were added to homogenates of both control and cortisone-treated embryos. Nucleic acid determinations were then carried out on these samples and the per cent recovery calculated.

Total Nitrogen and Protein Nitrogen: Nitrogen determinations were carried out on aliquots of the whole liver homogenate and the protein fraction according to the procedure of Koch and McMeekin (19). The total nitrogen content of the liver was determined from a 1.0 ml. aliquot of a 10% dilution of the liver homogenate. A 1.0 ml. aliquot of the protein fraction was used to determine the protein nitrogen content of the liver.

Glycogen and Fat: The glycogen content of the livers was determined using anthrone reagent according to the procedure suggested by Seifter *et al.* (34). Histological sections of the livers of both

control and treated chicks were examined for fat content by staining with Sudan Black according to the procedure of Baker (3).

Isolation of Nuclei: Nuclei were isolated from liver homogenates of control and treated embryos according to the procedure of Hogeboom *et al.* (14). Using the method already outlined, counts were made of the nuclei of the original homogenate and of isolated nuclei, and DNA determinations were done on the original homogenate and on the isolated nuclei.

Results

Body Weight and liver weight: Cortisone caused a decrease in body weight and in liver weight of the treated embryos. Body weights of treated chicks are significantly lower than those of controls after eleven days of incubation (Table 1). Liver weights are significantly lower in treated embryos after ten days of incubation (three days after cortisone administration).

Body weights and liver weights of both control and treated chicks show a daily increase throughout the interval studied, with the ex-

TABLE 1
Body Weights and Liver Weights of Control and Cortisone-treated Chick Embryos.

Age	Number of samples		Body weight in grams		Liver weight in mgm.	
	C	CT	C	CT	C	CT
9	5	5	1.7 ± 0.05	1.7 ± 0.03	23.0 ± 0.9	21.4 ± 0.7
10	5	5	2.7 ± 0.05	2.6 ± 0.1	42.0 ± 0.2	40.0 ± 0.2
11	5	5	3.9 ± 0.1	3.6 ± 0.1	62.0 ± 2.5	47.0 ± 2.3
12	8	6	6.1 ± 0.6	4.5 ± 0.2	106.0 ± 1.6	65.0 ± 3.7
13	8	11	8.3 ± 0.2	6.7 ± 0.2	155.0 ± 4.2	114.0 ± 6.4
14	8	10	12.4 ± 0.4	7.9 ± 0.2	218.0 ± 9.4	115.0 ± 9.2
15	9	11	14.6 ± 0.3	9.9 ± 0.8	294.0 ± 5.6	177.0 ± 17.5
16	9	8	17.4 ± 0.3	12.9 ± 0.7	369.0 ± 7.9	260.0 ± 22.5
17	9	8	20.7 ± 1.0	16.4 ± 1.0	473.0 ± 21.0	329.0 ± 23.6
18	9	8	24.2 ± 1.0	18.8 ± 1.1	502.0 ± 21.8	344.0 ± 24.6
19	7	8	30.4 ± 0.9	20.9 ± 3.1	557.0 ± 3.0	328.0 ± 37.0

C Controls
CT Cortisone-treated

ception that the liver weight of treated chicks does not increase between 13 and 14 days. The ratio liver weight/body weight is significantly lower in treated chicks after ten days of incubation.

Cells: The number of cells per unit weight is significantly reduced in the livers of the treated chicks at twelve and thirteen days of incubation ($p < 0.01$). Except for these two days there are no statistically significant differences on a unit weight basis throughout the interval studied. The number of cells per liver is higher in the control chicks after ten days of incubation (Table 2).

TABLE 2
Cells Per Unit Weight of Liver and Per Liver.

Age	Nuclei/mgm.		Cells per liver (nuclei × 10^3)	
	C	CT	C	CT
9	915,000 ± 66,000	907,000 ± 53,000	21,000	19,000
10	871,000 ± 28,000	824,000 ± 21,000	38,000	33,000
11	863,000 ± 38,000	918,000 ± 74,000	54,000	43,000
12	961,000 ± 27,000	760,000 ± 15,000	102,000	49,000
13	1,060,000 ± 54,200	787,000 ± 34,000	164,000	90,000
14	876,000 ± 62,000	904,000 ± 33,000	191,000	104,000
15	844,000 ± 35,000	958,000 ± 36,000	248,000	170,000
16	934,000 ± 53,000	863,000 ± 45,000	345,000	224,000
17	898,000 ± 36,000	857,000 ± 43,000	425,000	282,000
18	804,000 ± 20,000	825,000 ± 27,000	404,000	283,000
19	776,000 ± 38,000	891,000 ± 20,000	432,000	292,000

C Control
CT Cortisone-treated

Nucleic acids: In the nuclear isolation experiments an average recovery of 88.0 ± 2.7% of the DNAP of the homogenate was recovered in the nuclear fraction.

On the basis of cell counts of the homogenate and of the nuclear fraction, an average of 91.1 ± 3.4% of the cells of the homogenate were recovered on the nuclear fraction. The ratio of DNAP per nucleus in the isolated nuclei to DNAP per cell in the homogenate was 0.966 ± 0.037.

Cortisone causes only minor changes in the nucleic acid content of liver from treated embryos on a unit weight and cellular basis (Tables 3 & 4). The amount of DNAP is greater in the livers of treated embryos at thirteen days on a cellular basis and at nineteen days on a unit weight basis. Since at nineteen days there are slightly more cells per unit weight in the livers of treated embryos, the amount of DNAP per cell is approximately the same as that of the controls.

TABLE 3
DNAP Per Unit Weight of Liver, Per Cell and Per Liver.

Age	µgm/100 mgm		µgm × 10^{-7}/cell		µgm/organ	
	C	CT	C	CT	C	CT
9	42.0 ± 1.7	43.0 ± 1.4	4.6	4.7	9.7	9.2
10	31.0 ± 1.0	30.0 ± 1.0	3.6	3.7	13.2	12.0
11	43.0 ± 1.4	42.0 ± 4.5	5.0	4.6	26.7	20.0
12	35.0 ± 1.6	31.0 ± 0.8	3.6	4.1	37.0	20.1
13	34.0 ± 1.5	36.0 ± 2.6	3.2	4.6	52.7	41.0
14	34.0 ± 1.2	37.0 ± 1.6	3.7	4.1	74.1	42.6
15	38.0 ± 2.0	38.0 ± 2.0	4.5	4.0	111.7	67.1
16	38.0 ± 1.8	35.0 ± 1.4	4.1	4.1	140.2	91.0
17	32.0 ± 0.3	34.0 ± 1.9	3.6	4.0	151.4	111.9
18	33.0 ± 1.4	32.0 ± 1.3	4.1	3.9	165.7	109.9
19	31.0 ± 1.1	40.0 ± 1.9	4.0	4.5	172.7	131.2

C Control
CT Cortisone-treated

This illustrates the importance of calculating chemical constituents on a cellular basis. There are no differences apparent in the amounts of RNAP per cell and per unit weight in the livers of treated and control embryos.

Cortisone causes a reduction in the amount of nucleic acid per liver. Livers of treated embryos contain less DNAP and RNAP than those of controls after ten days of incubation. Since there is no significant increase in the amount per liver on the twelfth day of incubation there is apparently little new synthesis of either DNA or RNA.

The instantaneous rates of increase (k) of DNAP and of cells were

calculated on a percentage basis using the formula suggested by Brody (4).

$$k = \frac{100\ (\log_e W_2 - W_1)}{t}$$

where W_2 and W_1 are the final and initial amounts per liver respectively, and t is the time interval.

TABLE 4
RNAP Per Unit Weight of Liver, Per Cell and Per Liver.

Age	μgm/100 mgm		μgm × 10^{-7}/cell		μgm/organ	
	C	CT	C	CT	C	CT
9	121.0 ± 8.2	109.0 ± 3.3	13.2	12.0	28.6	23.4
10	77.0 ± 4.6	78.0 ± 3.6	8.8	9.5	32.7	31.0
11	115.0 ± 7.1	115.0 ± 8.5	13.3	12.5	71.5	54.1
12	86.0 ± 2.6	87.0 ± 2.6	9.0	11.4	90.8	56.3
13	81.0 ± 2.7	79.0 ± 2.9	7.6	10.0	125.6	90.1
14	79.0 ± 4.7	74.0 ± 3.0	9.0	8.2	172.2	85.1
15	74.0 ± 2.7	75.0 ± 4.9	8.8	7.8	217.6	132.4
16	82.0 ± 2.0	78.0 ± 5.6	8.8	8.9	302.6	202.9
17	79.0 ± 1.7	71.0 ± 1.9	8.8	8.3	373.7	233.6
18	71.0 ± 3.8	63.0 ± 1.6	8.8	7.6	356.4	216.4
19	68.0 ± 1.7	63.0 ± 1.4	8.8	7.1	378.8	206.6

C Control
CT Cortisone-treated

The results presented in Table 5 indicate that the rate of DNAP synthesis in the livers of control chicks is not constant throughout the interval studied. It is greatest at eleven days, fairly constant from days twelve through fifteen and then falls off rapidly by day nineteen.

The pattern is different in the livers of treated chicks and shows alternating high and low rates throughout the interval. On the eighteenth day there is no synthesis of DNAP and only a slight rate of synthesis on the twelfth and fourteenth days.

The instantaneous rate of increase of cells in the control livers does

not parallel that of DNAP. From days nine to thirteen a high rate of increase of DNAP is correlated with a low rate of increase in cell number and a high rate of increase in cell number is correlated with a low rate of increase in DNAP. After day thirteen this difference in rates is not as pronounced.

TABLE 5
Instantaneous Rates of Increase (k) of DNAP and Cells Per Liver (Expressed as Percentages)

Day	DNAP per liver		Cells per liver	
	C	CT	C	CT
9-10	29.3	29.0	59.3	55.2
10-11	73.0	51.0	35.1	26.5
11-12	31.5	5.0	63.5	13.0
12-13	35.9	62.2	47.4	60.8
13-14	33.4	4.7	15.2	14.4
14-15	41.4	45.8	26.1	49.1
15-16	22.3	29.1	33.0	27.6
16-17	7.5	20.8	20.9	50.6
17-18	9.5	0.0	0.0	0.0
18-19	4.1	15.7	1.6	3.4

C Control
CT Cortisone-treated

The livers of treated embryos show a similar pattern on the tenth, eleventh and seventeenth days. On the other days the rates of both DNAP synthesis and increase in number of cells are similar.

Total nitrogen and protein nitrogen: Cortisone exerts little effect on the total nitrogen and protein nitrogen content of embryonic livers on a unit weight or cellular basis (Tables 6 & 7). The amount of protein nitrogen is significantly lower on a unit weight basis in livers of treated embryos at thirteen days, but this is not reflected in a decreased amount per cell.

There is a statistically significant decrease in the amounts of total and protein nitrogen in the livers of treated chicks from thirteen through nineteen days of incubation.

Glycogen: The mean amount of glycogen is consistently higher in the livers of treated chicks but the variation is so great that no statistically significant differences are apparent until eighteen days. On this day the mean amount of glycogen per unit weight, per cell and per liver in the treated chicks is more than twice that in the controls. There is, however, a tendency for the treated livers to have more glycogen per unit weight and per cell (Table 8).

Fat: No difference in fat content was observed in histological sec-

TABLE 6
Total Nitrogen Per Unit Weight of Liver, Per Cell and Per Liver

Age	μgm/mgm		μgm × 10^{-7}/cell		mgm/organ	
	C	CT	C	CT	C	CT
9	21.2 ± 4.0	26.6 ± 0.6	231	290	0.49	0.57
10	20.0 ± 1.2	17.8 ± 0.6	304	216	0.85	0.71
11	28.0 ± 0.8	28.5 ± 0.9	325	310	1.74	1.34
12	16.2 ± 0.2	19.3 ± 0.7	168	254	1.71	1.60
13	21.6 ± 1.6	30.3 ± 1.2	204	258	3.35	2.31
14	22.7 ± 1.0	22.4 ± 1.4	259	248	4.95	2.58
15	23.8 ± 0.9	19.7 ± 1.2	282	206	7.00	3.48
16	25.4 ± 0.2	24.1 ± 0.8	272	278	9.37	6.27
17	25.7 ± 0.5	24.7 ± 0.8	286	288	12.16	8.15
18	23.8 ± 2.0	22.4 ± 1.2	296	272	11.95	7.70
19	25.5 ± 0.5	23.1 ± 1.2	328	260	14.20	7.57

C Control
CT Cortisone-treated

TABLE 7
Protein Nitrogen Per Unit Weight of Liver, Per Cell and Per Liver

Age	μgm/mgm		μgm × 10^{-7}/cell		mgm/organ	
	C	CT	C	CT	C	CT
9	18.2 ± 1.6	22.5 ± 0.8	198	248	0.42	0.48
10	15.5 ± 0.6	15.2 ± 0.3	177	184	0.67	0.60
11	19.0 ± 1.3	18.0 ± 1.8	220	196	1.18	0.86
12	14.6 ± 1.2	16.0 ± 0.7	153	210	1.55	1.04
13	17.9 ± 0.7	13.9 ± 1.1	169	176	2.77	1.58
14	18.3 ± 1.0	15.8 ± 1.1	209	175	3.99	1.82
15	17.4 ± 0.3	15.2 ± 1.2	206	159	5.12	2.69
16	18.4 ± 0.8	18.3 ± 0.6	197	212	6.79	4.50
17	18.8 ± 1.1	18.5 ± 0.6	210	214	8.85	6.10
18	15.9 ± 0.7	19.0 ± 1.0	198	230	7.98	6.52
19	18.9 ± 1.3	14.8 ± 2.0	248	166	10.52	4.85

C Control
CT Cortisone-treated

tions of control and cortisone-treated chick livers examined at two day intervals from twelve through eighteen days. Although the amount of fat stained by Sudan Black increased from twelve to eighteen days in all animals, no detectable differences were found between the control and experimental series.

TABLE 8
Glycogen Per Unit Weight of Liver, Per Cell and Per Liver

Age	mgm/100 mgm		μgm × 10^{-6}/cell		mgm/organ	
	C	CT	C	CT	C	CT
12	0.88 ±0.17	1.43 ± 0.34	9.3	18.8	0.93	0.93
14	1.54 ±0.25	3.06 ± 0.92	17.5	33.9	3.36	3.50
16	2.13 ±0.63	3.54 ± 0.83	22.1	41.0	7.88	9.20
18	2.85 ±0.84	10.40 ± 1.00	35.4	116.0	14.30	35.80

C Control
CT Cortisone-treated

Discussion

Cortisone was found to exert the following effects on the developing chick liver: The amounts of DNAP, RNAP, protein nitrogen and total nitrogen were significantly lower on a total weight basis in the treated livers after about ten days of incubation. The amounts of these substances per cell and per unit weight were generally the same in control and treated livers. The glycogen content was greater in the treated livers but no differences were noted in fat content.

The effects of cortisone on body weight and liver weight are first apparent at eleven to twelve days of incubation (three to four days after the injection of cortisone). At this time both body weight and liver weight are significantly lower than those of the controls. These results are in agreement with those of Karnofsky *et al.* (16), who reported that cortisone exerts its effect between ten and twelve days of incubation.

DNAP per nucleus: In the present study the amount of DNAP per nucleus was not constant throughout the incubation period in either the control or treated chicks. Since the data represent average amounts per nucleus the amount in individual nuclei may vary considerably from this figure. However, the claim of constancy for the amount of DNA per nucleus cannot be expected to apply to nuclei of a cell population of a rapidly growing organ such as embryonic

liver. It is generally stated (1, 11, 36) that the amount of DNA per nucleus doubles during interphase. Therefore, in the embryonic liver the average amount of DNAP per cell depends upon the rate at which DNAP is being accumulated in interphase nuclei and the number of interphase nuclei at any particular moment. The average values of 4.00×10^{-7} ugm./nucleus for the controls and 4.20×10^{-7} ugm. DNAP/nucleus for the treated chicks reported here are intermediate between the value of 2.35×10^{-7} ugm. DNAP/nucleus, reported by Leslie and Davidson (22) and the tetraploid amount, 4.70×10^{-7} ugm. These values are much lower than the average of 8.2×10^{-7} ugm. DNAP/nucleus in the wings and legs of developing chick embryos reported by Nowinski and Yushok (28).

It is of interest to note here the close agreement between two different methods of arriving at the amount of DNAP per nucleus. The results obtained from cell counts and DNAP in homogenates of the liver differed from those obtained from cell counts and DNAP in isolated nuclei of the livers by only 3.7%.

Cell division and synthesis of nucleic acids: The effect of cortisone on cell division and nucleic acid synthesis is not clearly understood. Cavallero *et al.* (6) concluded that cortisone inhibited the synthesis of both RNA and protein in chick embryos. These conclusions were based on data pooled from homogenates of the entire embryo. Several authors have reported decreased synthesis of RNA in mammals after cortisone treatment (3, 17, 26, 31). Einhorn *et al.* (9) reported an inhibition in the restoration of cells in regenerating livers of rats treated with cortisone and suggested that this inhibition is due to a block in nucleic acid synthesis. Roberts *et al.* (30) reported somewhat similar results from regenerating livers of mice treated with cortisone.

In the present study at least two different hypotheses are possible to account for the reduction in size of the livers of treated chicks: (1) Cortisone could inhibit nucleic acid synthesis, causing a decrease in cell division and/or a decrease in protein synthesis, or (2) The primary effect of cortisone could be inhibition of cell division rather than inhibition of nucleic acid synthesis.

In general, it is expected that an inhibition in synthesis of a substance will be reflected in a decreased amount of that substance per unit weight, and possibly per cell. In the present study, there are no statistically significant decreases in the amounts of DNAP per unit weight or per cell in the livers of treated embryos. The amount of

RNAP is significantly decreased on a unit weight basis in the treated series at seventeen days, but this is considerably later than the reduction in size of the liver. There are no significant differences in the amounts per cell at any time during the period studied. Consequently, there is no evidence that cortisone has caused an inhibition of synthesis of either DNA or RNA in the livers of treated chicks.

The instantaneous rates of DNA accumulation offer the best means of determining the extent of new synthesis of DNA. The rates of DNA accumulation do not parallel those of increase in cell number in control chicks from days nine to thirteen (Figure 5). It would appear that DNA is synthesized during one day and cell division takes place during the next. There is a rhythmicity of DNA synthesis and cell division with peaks of DNA synthesis followed by peaks of cell division. This is true only during the time of most rapid cell division. These results are similar to those reported by Price and Laird (29) in studies on regenerating rat liver.

In the treated embryos the rates of DNA synthesis and cell division are similar to those of controls until the twelfth day, when the rhythmicity is interrupted. It appears that the major action of cortisone in this case may be a blocking of mitosis rather than a blocking of DNA synthesis. During the eleventh day of incubation the rate of cell division is somewhat impeded in the treated chicks. This is probably sufficient to explain the differences in liver weights at this time. On this day the rate of DNA synthesis in the livers of control chicks is the highest of any day of the period studied and is followed by a high rate of cell division. In the livers of treated chicks the rate of DNA synthesis is lower, possibly because of a cortisone block, but the more pronounced effect is on the rate of cell division during the following day. At this time it is the lowest of any day from nine through seventeen and is less than one-fourth the rate of mitosis in control livers.

There is little synthesis of liver DNA in treated chicks during the twelfth day but this is probably not due primarily to inhibition by cortisone, but to the fact that the DNA has already been synthesized during the preceding day but the cells have not divided. On the thirteenth day the rates of DNA synthesis and cell division are both greater in the livers of treated chicks than livers of controls. During the fourteenth day mitosis is again impeded in the livers of treated chicks. The rate of DNA synthesis is, therefore, low on this day and the rate of cell division is high on the following day.

In general, after the fourteenth day the rates of liver cell division and DNA synthesis are somewhat higher in treated chicks than in controls but the absolute values per liver of each are less after ten days. It appears that the initial decrease in the rate of cell multiplication during the tenth day results in a reduction in the total number of cells per liver which cannot be repaired thereafter. The most important defect in these livers is undoubtedly a blocking of cell division during the twelfth and fourteenth days of incubation. The block at twelve days may be related to a slight decrease in the rate of synthesis of DNA during the eleventh day of incubation and is manifested at the time of most rapid cell proliferation in the livers of control chicks.

There is no evidence of any inhibition in synthesis of liver RNA in treated chicks. In general, the rates of synthesis of liver RNA parallel those of liver DNA, and the decrease in total content of liver RNA in treated chicks after ten days can be adequately accounted for on the basis of a reduced number of cells as the results of a decreased mitotic rate. The same explanation suffices for the decreased amounts of total nitrogen and protein nitrogen in the livers of treated chicks.

Glycogen: It has been known for a long time that corticosteroids may cause an increased deposition of liver glycogen (13, 23). Sames and Leathem (32) reported an increase in liver glycogen in cortisone-treated chicks after day eleven. The present study confirms this report, but the increase in liver glycogen in treated chicks is not statistically significant until eighteen days of incubation. There is, however, a trend toward glycogen storage, as shown in Table 8. The values for liver glycogen in control chicks are in excellent agreement with those reported by Lee (21).

In addition to increasing gluconeogenesis, the glucocorticoid hormones are believed to decrease carbohydrate usage (7, 24, 25). It is possible that the decrease in liver size and in liver cell number in treated chicks is due to such a block in carbohydrate usage, although Bullough (5) was unable to determine the precise point of action of cortisone in carbohydrate utilization in mitosis in mouse epidermis, and concluded that it interferes with energy production at more than one point. However, conclusions based on *in vitro* studies of adult mammalian tissue may not be applicable to embryonic tissue studied *in vivo*.

Fats: There are conflicting reports concerning the effects of corti-

sone on fat. Kochakian and Robertson (18) reported a decrease in liver fat in mice treated with cortisone, whereas Ingle (15) found a decrease in liver fat after adrenalectomy. Roberts *et al.* (30) and Einhorn *et al.* (9) reported an increase in liver fat in regenerating liver of mice and rats treated with cortisone. In the present study no differences in fat content were noticed, and it is believed that marked changes in fat content do not occur in the livers of cortisone-treated chicks.

Summary

The effects of cortisone on the size, cell number and amounts of various chemical constituents of embryonic chick liver at daily intervals from nine through nineteen days have been studied. The size, weight and cell number of livers of treated chicks were significantly reduced. There was no significant reduction in the amounts of nucleic acids, protein nitrogen, total nitrogen or fat in livers of treated chicks on a unit weight or per cell basis. The amount of liver glycogen was significantly increased in treated chicks.

Reasons are offered for believing that the reduction in cell number and the accompanying decrease in liver size and total nucleic acid and nitrogen content of livers of treated chicks are due mainly to a block in mitosis that is not associated primarily with inhibition of nucleic acid synthesis.

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THE DEMONSTRATION OF A FACTOR CONCERNED WITH THE INITIATION OF ANTHERIDIA IN POLYPODIACEOUS FERNS

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INTRODUCTION

Döpp (1950) reported that an extract from prothalli of the fern *Pteridium aquilinum* (L.) Kuhn stimulated antheridium production in young prothalli of *Pteridium aquilinum* and hastened the onset of antheridium formation in prothalli of *Dryopteris Filix-mas* (L.) Schott by several weeks. The active substance was readily soluble in water but sparingly soluble in ether and acetone. It was partially destroyed by hot ammonium hydroxide and completely destroyed by hot hydrochloric acid and by hot hydrogen peroxide.

Döpp envisaged the possibility that the extract-induced antheridia were initiated by way of unspecific growth inhibition. This interpretation was prompted by his observation that the extract inhibited growth. It was also compatible with the observations of earlier investigators (see *e.g.*, Prantl, 1881) that "male" prothalli are smaller than "female" prothalli and that conditions interfering with growth, *e.g.*, low light intensity, crowding and poor mineral supply, favor the production of antheridia over that of archegonia.

The significance of antheridium induction by the extract clearly cannot be ascertained until this interpretation is either confirmed or refuted by evidence leading to the alternative interpretation that the biologically active factor is a normal metabolite of fern gametophytes and specifically concerned with the induction of antheridia.

An attempt has therefore been made to investigate this problem. Attention has also been directed toward devising a reliable assay system to determine additional properties of the biologically active factor and to establish a preliminary activity spectrum of the antheridium-inducing factor among polypodiaceous ferns.

Methods

Sterilization and inoculation of the spores. About 3 mm^3 of spore material was put into a 5 ml glass tube and shaken with aerosol water (two drops of 25 per cent aerosol OT per 100 ml of water) until the spores were in suspension. The suspension was filtered through hard filter paper, the wet spore mass scraped off with a small spatula and transferred to the bottom of a sterile 5 ml glass tube. Two drops of hypochlorite solution (Clorox 1:10, commercial product) were then added to the spores. After 10 seconds sterilization was stopped by the addition of ca. 4 ml of sterile water. The resulting suspension was transferred to a 20 ml test tube and diluted to a final volume of 10 ml. By means of small sterile pipettes, two to six drops of this suspension were added to the culturing flasks, the number of drops depending on the size of the culturing flask and the desired concentration of the inoculum. The suspension was distributed evenly over the agar surface by gently tilting the flask in all directions. (Wetting of the spores by aerosol water prior to sterilization shortened the time necessary for effective sterilization. If this was not done, a considerable number of spores were killed before sterilization was complete.)

Method of culture. The medium described by Moore (1903), supplemented with Hoagland's A-Z solution of microelements, was used throughout the investigation. The iron was added as ferric tartrate (5 mg/1). The fern material used for the preparation of extracts was grown in 125 ml flasks containing 33 ml of medium solidified by one per cent agar. The assays were conducted in 50 ml Erlenmeyer flasks containing 12 ml of agar-solidified medium. The cultures were grown at 23 $\pm$ 0.5°C under continuous illumination by cool white fluorescent lamps at an intensity of ca. 200 f.c.

Assay. Gametophytes of *Pteridium aquilinum* were confirmed to be a rich source of the antheridium-inducing factor. They were harvested after growing on agar-solidified medium for 7 weeks, put into a mortar and, after the addition of fresh medium (volume in ml equal to half the fresh weight of the prothalli in gm), thoroughly ground. After centrifugation for 20 minutes at 6000 r.p.m., the supernatant was decanted and ready for assay. More recently liquid medium, on which *Pteridium* prothalli had been growing for 7 weeks, was found to contain the antheridial factor in almost as high a concentration as the prothalli themselves. Since the culture fluid inhibited growth of the assay gametophytes less than the extract, and because its

preparation was simpler, it was used as the source of the antheridial factor in all subsequent experiments.

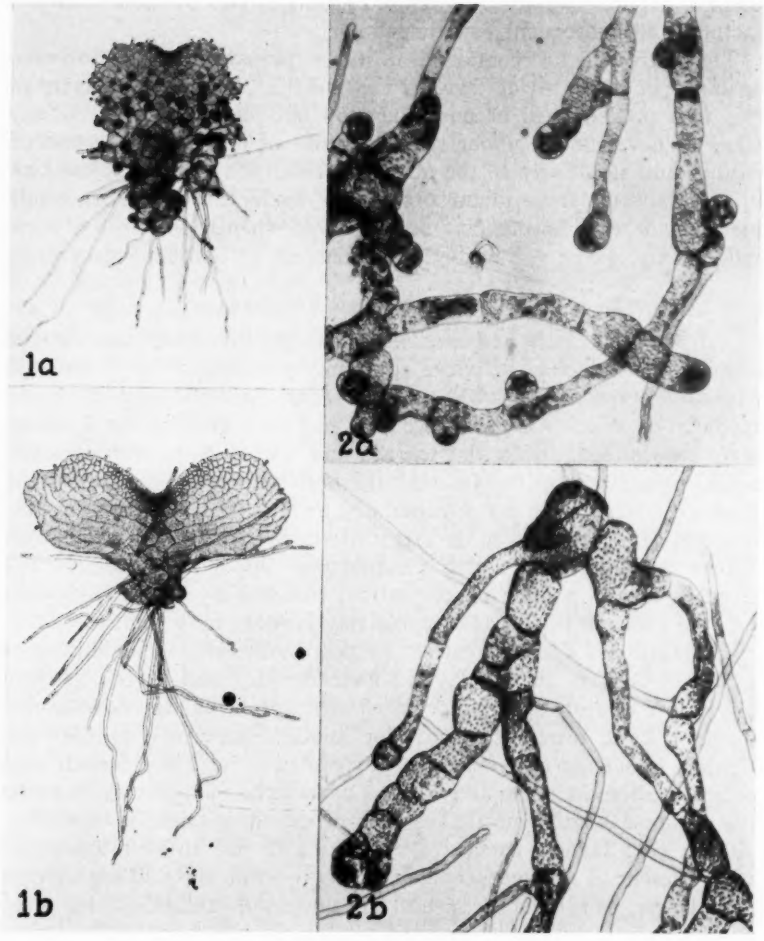
The active factor contained in these preparations was assayed against gametophytes of *Onoclea sensibilis* L. The gametophytes of this fern proved ideal because they did not form antheridia at any stage of development under our conditions of culture but responded readily and sensitively to the addition of extract. The complete lack of spontaneous antheridium production made it possible to safely identify antheridium-inducing activity even when it was so low as to induce only one or two antheridia in one out of ten gametophytes.

Experiments and Results

Activity of extract and medium. The dilution endpoints for the antheridium-inducing activities present in extracts of 7-week-old *Pteridium* prothalli (grown on solid agar medium) and in liquid medium, on which *Pteridium* prothalli had been growing for 7 weeks, were determined. Both the extract and the medium were assayed against gametophytes of *Onoclea sensibilis* either by inoculating the spores directly on the factor-containing medium or by transferring 12-day-old gametophytes on it (see "Methods" for details of assay). Either source of the active material was applied at one-half full strength as the highest concentration, followed by 1/10, 1/50, etc., *i.e.*, the concentration was progressively lowered by a factor of 5.

It was found that the extract induced antheridia to a dilution of 1:31,250 both in the 12-day-old gametophytes and in the gametophytes grown from spores inoculated directly on the factor-containing medium. The lowest concentration induced only one to four antheridia in one out of every five assay prothalli, but the demonstration of antheridium-inducing activity was nevertheless unequivocal because not a single control prothallus had produced a single antheridium. At the next highest concentration, 1: 6,250, one to four antheridia were present in nearly every gametophyte, while the still higher concentrations had induced several to numerous antheridia in every gametophyte. While these assays were routinely read 12 days after inoculation, antheridia initials could be clearly discerned 6 days after inoculation. In six assays a second reading was taken after 16 days. In two of them activity assayed to 1:156,250, *i.e.*, to a dilution five times lower than after 12 days.

Liquid medium on which *Pteridium* prothalli had been growing for



FIGURES 1-2

1a) Gametophyte of *Onoclea sensibilis*, 19 days old, 33 X, grown in the presence of *Pteridium* medium 1:250. 1b) Same as 1a, except gametophyte grown in the absence of *Pteridium* medium. 2a) Gametophyte of *Onoclea sensibilis* 14 days old, grown in red light, and in the presence of *Pteridium* medium 1:250, 106 X. 2b) Same as 2a, except gametophyte grown in the absence of *Pteridium* medium. (Photographs by J. A. Carlile.)

7 weeks could be diluted either 31,250 or 6,250 times and still retain activity; the higher activity was rather regularly obtained if at harvest time the prothalli had covered the surface of the medium. Under these circumstances growth was inhibited less than when extract was used. The liquid medium was therefore used as the source of the antheridium-inducing factor in all subsequent experiments.

Figure 1a shows an *Onoclea* prothallus studded with the numerous antheridia induced by *Pteridium* medium at 1/250 full strength, while the control prothallus does not bear a single antheridium (Figure 1b). Figures 2a and 2b show *Onoclea* gametophytes grown in red light (obtained by combining red G.E. fluorescent lamps with Perspex plastic "red 400") which causes them to grow in a filamentous fashion (Klebs, 1917). Under such conditions *Pteridium* medium again induced the formation of numerous antheridia (Figure 2a). None were present in the controls (Figure 2b). Figure 3 shows antheridia induced in red

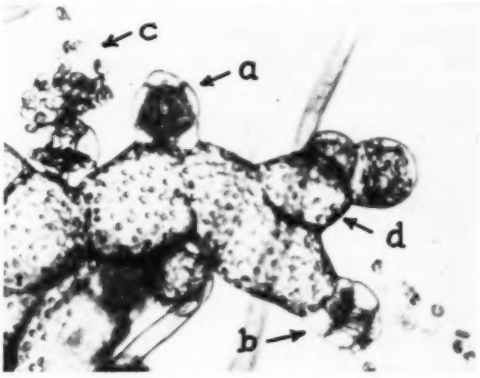


FIGURE 3
Gametophytes of *Onoclea sensibilis*, 14 days old, 200X, grown in the presence of *Pteridium* medium 1:250. (Photograph by J. A. Carlile.)

light at higher magnification. This picture demonstrates that the structure of such induced antheridia does not in any way deviate from that of spontaneously formed antheridia. Thus, starting at the base, the characteristically funnel-shaped stalk cell is followed by the ring cell (its shape may be visualized by comparing its appearance in the emptied antheridium shown by arrow "b" with its appearance in the

intact antheridium shown by arrow "a"). The ring cell is topped by the cap cell. The sperm cells may be seen in several of the antheridia shown in this picture.

Addition of water to mature induced antheridia promptly released the sperm cells (arrow "c") which soon began to swim around. Their motility, and their ability to bring about the formation of sporophytes in antheridia-free cultures of *Onoclea* gametophytes, must be considered evidence that they are functional.

Properties of the active factor. Döpp's finding that the factor is readily soluble in water but sparingly soluble in ether was confirmed; continuous extraction with ether of *Pteridium* medium adjusted to pH 3 for 4 hours removed about half the activity, 20 hours being required for its complete removal. The activity resisted boiling at the pH of the medium or the extract, as found by Döpp. It proved to be stable to autoclaving for 15 minutes at 15 lbs. pressure as well as to boiling for 10 minutes at pH 2. Boiling for the same length of time at pH 12, however, destroyed about 80 per cent of its activity. The active material dialyzed freely through cellophane and was adsorbed on charcoal from which it could be eluted with methanol. These results were obtained by means of dilution series, the dilutions being progressively lowered by a factor of 5. The above statements regarding stability are therefore correct only within the limits imposed by the spacing of the dilutions. Also, the mentioned properties are not necessarily those of the pure compound.

Specificity of the active factor. The hypothesis that the extract induces antheridia by way of unspecific growth inhibition (see Introduction) would become untenable if it were demonstrated that the active factor can induce antheridia without at the same time inhibiting growth. The observation that liquid *Pteridium* medium with antheridium-inducing activity equal to that of extract did not noticeably inhibit growth pointed in this direction. The question of whether such medium inhibits at all was answered by quantitatve measurements of growth.

In a first approach the effect of *Pteridium* medium on growth by cell division in gametophytes of *Pteridium aquilinum* and *Onoclea sensibilis* was determined. Spores of these two ferns were inoculated directly on medium into which *Pteridium* medium had been incorporated at 1/10 and 1/50 full strength. Cell counts were taken when the first antheridia had been initiated, *i.e.,* 5 days after inoculation in the case

of *Pteridium aquilinum* and 7 days after inoculation in the case of *Onoclea sensibilis*. The results are presented in Table 1. Each value is based on cell counts on 150 gametophytes, 25 randomly chosen gametophytes being counted in each of 6 flasks (the standard error represents the spread between the averages obtained in each of the 6 flasks). The average cell number present in the antheridia initials is given in parentheses, while the preceding value lists the total number of cells (vegetative cells plus the cells present in the antheridia).

Table 1 shows that the control gametophytes contain a smaller number of cells than the gametophytes grown on *Pteridium* medium

TABLE 1

	Number cells	
	Pteridium aquilinum	*Onoclea sensibilis*
Control	12.2 ± .4	9.4 ± .2
Pteridium medium 1/10	16.5 (7.1) ± .5	11.4 (2.9) ± .2
Pteridium medium 1/50	16.7 (6.6) ± .3	10.7 (2.5) ± .4

(the differences are significant at the 10 per cent level). Thus, *Pteridium* medium promotes rather than inhibits growth by cell division. It remains to be determined whether this promotion is brought about by the antheridium-inducing factor or another constituent of the *Pteridium* medium.

While increasing the total number of cells, the *Pteridium* medium brought about a reduction in the number of vegetative cells. This is readily understood if we consider that the antheridium-inducing *Pteridium* medium diverted part of the growth potential from the formation of vegetative cells to the formation of antheridial cells.

It, nevertheless, appeared desirable to study the effect of the antheridial factor on vegetative growth in the absence of antheridium production. This was not feasible with the above system because the very first signs of antheridium production were in some cases visible at the three or four cell stage. A suitable test system became available with the observation that 3-week-old *Onoclea* prothalli were no longer sensitive to the antheridial factor; in such prothalli *Pteridium* medium did not induce any antheridia even if supplied at one-half full strength,

i.e., a concentration 15,000 times higher than the concentration necessary to induce antheridia in gametophytes only one week younger. This result will be presented in greater detail and its significance discussed in a subsequent paper.

Onoclea prothalli were grown for 21 days without an added source of antheridial factor and then transferred to solid medium without antheridial factor or with the antheridial factor supplied as *Pteridium* medium at 1/10 or 1/50 full strength. Five prothalli were transferred to each flask. Growth, in terms of fresh weight and surface area, was measured 12 days after transfer. The results are listed in Table 2,

TABLE 2

	Index surface area	Fresh weight
Control	52.3 ± 1.9	6.90 ± .24 mg
Pteridium medium 1/10	53.9 ± 1.8	6.80 ± .19 mg
Pteridium medium 1/50	55.6 ± 1.8	6.82 ± .18 mg

each value being based on measurements on 60 gametophytes. The standard error again represents the spread between the averages obtained in each flask. The index for surface area was obtained by multiplying distances a and b (measured in mm) of Figure 4. The differences between the values obtained for gametophytes grown in the presence of the antheridial factor and those obtained for gametophytes grown in its absence are not significant at the 10 per cent level. Thus the ability of the *Pteridium* extract to induce antheridia must be considered independent of its growth-inhibiting action. Instead, the results suggest that the active factor is a natural metabolite of *Pteridium* gametophytes and specifically concerned with the induction of antheridia.

If this is the case, then the factor might be present in effective concentrations in gametophytes of ferns that form antheridia under the prevailing conditions of culture (such as *Pteridium aquilinum* and *Blechnum gibbum*) but lacking in gametophytes of ferns that do not form antheridia under these same conditions (such as *Onoclea sensibilis* and *Thelypteris* sp.). Extracts were prepared from 9-week-old gametophytes of *Blechnum gibbum* and from 7-week-old gametophytes of the more quickly growing three other fern species. The

activities of these solutions were again assayed against prothalli of *Onoclea sensibilis* and read 12 days after inoculation.

The dilution endpoints for the extracts from *Blechnum gibbum* and *Pteridium aquilinum* were both 1:31,250. The *Thelypteris* extract was active to a dilution of 1:10, while the *Onoclea* extract was inactive even at the highest concentration (one-half full strength). If, however, the assay time was lengthened to 17 days, the *Onoclea* extract showed some activity in two out of five assays, the dilution endpoint being 1:2 in either case. Thus, at least 15,000 times more activity is found in the gametophytes of either antheridia-forming fern (*Blechnum gibbum, Pteridium aquilinum*) than in the antheridia-free gametophytes of *Onoclea sensibilis,* and 3,000 more than in the equally antheridia-free gametophytes of *Thelypteris* sp. This striking correlation leaves little doubt as to the specific association of the active factor with antheridium production.

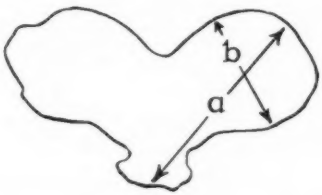


FIGURE 4

Outline of *Onoclea* gametophyte. Distances a and b were used in the calculation of the index for surface area.

Activity spectrum. It has thus far been demonstrated that the *Pteridium* factor significantly hastens the onset of antheridium production in gametophytes of *Pteridium aquilinum* and brings about the formation of antheridia in *Onoclea sensibilis* which does not form them spontaneously under the conditions of the experiment. An extension of the studies to other ferns was considered desirable at this point both to learn whether the *Pteridium* factor stimulated antheridium production in other ferns and to obtain information as to whether this factor, or a similar one, was present in the gametophytes of other

ferns. All species involved in this investigation belonged to the family Polypodiaceae.

The tests for activity of the *Pteridium* factor toward the gametophytes of other ferns were conducted in one of two ways. If growth of the fern to be tested was rapid, the spores were inoculated directly on the factor-containing medium (supplied as 1/50 full strength *Pteridium* medium). If, on the other hand, growth was slow, the prothalli were pre-grown for 2 to 3 weeks on factor-less medium; 1 ml of 1:1 *Pteridium* medium was then added to the agar surface on which the test prothalli were growing, the controls receiving the same amount of fresh medium. The following seven ferns were used in this survey (subgroups of the family Polypodiaceae added in parentheses): *Athyrium thelypteroides* (Michaux) Desvaux (Asplenioids); *Thelypteris hexanoptera* (Michaux) Weatherby, *Polystichum acrostichoides* (Michaux) Schott, *Thelypteris* sp. (Dryopteroids); *Woodwardia areolata* (L.) Moore (Blechnoids); *Pteretis pensylvanica* (Willdenow) Fern (Onocleoids); *Polypodium aureum* L. (affinity unclear).

The *Pteridium* factor clearly stimulated antheridium production in six out of these seven fern species, *Polypodium aureum* being the only exception. Prothalli of this fern not only failed to produce antheridia in the presence of the *Pteridium* factor but also in its absence throughout the 9 weeks they were under observation. On the other hand, the *Pteridium* factor induced numerous antheridia in *Thelypteris* sp. even though this fern did not produce any antheridia spontaneously at any stage of its development. Prothalli of the remaining five ferns all formed antheridia spontaneously. Addition of the *Pteridium* factor, however, hastened the onset of antheridium production in the various species by a minimum of 4 weeks and a maximum of 8 weeks.

Attempts to demonstrate the *Pteridium* factor in other ferns were restricted to *Thelypteris hexanoptera* and *Blechnum gibbum*. Extracts were obtained from 10-week-old gametophytes of *Blechnum gibbum* and 7-week-old gametophytes of *Thelypteris hexanoptera*. Numerous antheridia were present in the cultures of both species. The *Blechnum* extract induced the formation of antheridia in gametophytes of *Onoclea sensibilis* to a dilution of 1:31,250, the *Thelypteris* extract to a dilution of 1:6,250.

Discussion

Döpp's report that gametophytes of *Pteridium aquilinum* form antheridia a few days earlier if grown in the presence of extract from older gametophytes was confirmed. In other ferns, though, the time lag between induced and spontaneous antheridium formation was found to be greater, ranging from 4 to 8 weeks. Most remarkable of all was the finding that the extract induced antheridia in gametophytes of *Onoclea sensibilis* and *Thelypteris* sp. even though gametophytes of these two species did not form antheridia spontaneously at any stage of development. The *Onoclea* gametophyte proved ideal for the detection of antheridium-inducing activity and was therefore used in the sensitive and reliable assay method described above.

Extract from 7-week-old *Pteridium* gametophytes was demonstrated to be active to a dilution of 1:31,250 (Döpp's extract was active to a dilution of 1:100). These gametophytes thus contain the active factor far in excess of the concentration necessary for the induction of antheridia. Large amounts of the active material were also secreted into the medium, its activity being often as great as that of the extract. This high activity appears to be the result not only of relatively high concentrations of the active factor but also of its ability to induce antheridia at very low concentrations. The available data show in fact that it must be active at a concentration as low as 1.6×10^{-9}. The calculation is based upon the weight of the dry matter (10 mg) present in an ether extract containing the total activity of one liter of *Pteridium* medium that was active to a dilution of 1:6,250. Very likely the *Pteridium* factor accounts for only a small fraction of the dry matter, in which case it would be active at concentrations considerably below 1.6×10^{-9}.

This activity of the *Pteridium* factor at extremely low concentrations is compatible with the hypothesis that the factor is a natural metabolite concerned specifically with the induction of antheridia. Such a hypothesis, though, cannot be reconciled with the hypothesis that the extract induced antheridia by way of unspecific growth inhibition (see Introduction). Doubts regarding this hypothesis arose with the observation that *Pteridium* medium with antheridium-inducing activity equal to that of extract did not noticeably inhibit growth. This showed that at least part of the growth-inhibiting properties of the extract cannot be ascribed to the factor responsible for antheridi-

um induction. Measurements in terms of cell number, fresh weight, and surface area actually demonstrated that highly active concentrations of *Pteridium* medium did not inhibit growth.

Further investigations showed that extracts from prothalli of two ferns that produced numerous antheridia under the prevailing conditions of culture contained antheridium-inducing activities 3,000 to 15,000 times higher than extracts from prothalli of two ferns that did not form antheridia under these same conditions of culture. This result left little doubt as to the specific association of the active factor with antheridium production.

The hypothesis that the extract induces antheridia by way of unspecific growth inhibition was thus proved invalid on two counts, first by the demonstration that the active factor induced antheridia without inhibiting growth, and second, by the demonstration of its specific association with antheridium production. Instead, the results strongly support the view that the active factor is a normal metabolite of gametophytes, specifically concerned with the induction of antheridia.

Even though it was demonstrated that antheridium induction need not be accompanied by growth inhibition, the fact remains that the "male" prothalli are strikingly smaller than the "female" prothalli. It may be hypothesized that this smaller size of the "male" gametophyte is related to the investment of a large part of its growth potential into the formation of antheridia. After releasing the sperm cells, these antheridia disintegrate and do not, therefore, appear in any subsequent measurement of growth. True, the corresponding thing happens in the "female" gametophyte where the archegonia also disintegrate after a while. However, the investment of growth potential into the formation of archegonia would appear to be smaller. First, because every cell of the "male" gametophyte (except probably the few rhizoid-bearing cells) can form antheridia, while only a fraction of the cells making up the "female" gametophyte are involved in the formation of archegonia. Second, because a given cell of the "male" gametophyte may give rise to more than one antheridium, while a vegetative cell involved in the formation of an archegonium will not take part in this process again.

A cell bearing two antheridia (one structurally complete, the other an early initial) can be seen in Figure 3 arrow "d". It pictures antheridia induced on the filamentous gametophytes formed in red light.

Such cells—one to many, depending on the size of the gametophyte—may also be seen on prothalli grown in white light. More rarely, cells bearing three antheridia were observed. Cells bearing more than one antheridium were always among those that were largest and most mature. Antheridia were not, however, initiated at the same time, as shown by the considerable differences in their stages of development. The smaller cells probably cannot initiate a second antheridium before the first one has disintegrated.

Most of the experiments were carried out on two ferns, *Pteridium aquilinum* and *Onoclea sensibilis,* the gametophyte of the former serving as the source of the antheridial factor, while the gametophyte of the latter fern was used as the assay prothallus. Subsequent investigations demonstrated that this factor, or a similar one, was present and/or functional in a considerable number of other polypodiaceous ferns. Thus, antheridium-inducing activity was demonstrated in extracts from gametophytes of *Thelypteris hexanoptera, Blechnum gibbum* and *Thelypteris* sp., while the *Pteridium* extract was shown to induce antheridia in six further polypodiaceous ferns, which in turn belonged to five genera. Adding *Pteridium aquilinum* itself, as well as *Athyrium Filix-mas* (Döpp, 1950) and *Onoclea sensibilis,* we arrive at a total of seven genera. These seven genera are representative of five subgroups of the family Polypodiaceae (Onocleoids, Blechnoids, Athyrioids, Asplenioids, Pteroids) out of the nine listed in Eames (1936). One polypodiaceous fern, *Polypodium aureum,* did not, however, produce any antheridia either spontaneously or in response to added antheridium-inducing factor. Recent observations have nevertheless indicated that this fern does form antheridia, both spontaneously and in response to added antheridial factor, if provided with a more suitable environment. It thus appears likely that the antheridial factor originally demonstrated in *Pteridium aquilinum* is present, and functional in antheridium formation, in all polypodiaceous ferns. Speculation as to its occurrence in other fern families is of considerable interest. It is of significance to recall in this connection that the family Polypodiaceae comprises the phylogenetically most recent ferns and is a highly unnatural grouping, *i.e.,* several of the subfamilies are thought to have arisen independently from various older stock. This greatly enhances the possibility that the biologically active factor concerned with antheridium induction is present and functional throughout the Filicinae.

Summary

Döpp's report that gametophytes of *Pteridium aquilinum* form antheridia a few days earlier if grown in the presence of extract from older gametophytes was confirmed. The same extract hastened the onset of antheridium formation in other polypodiaceous ferns by a minimum of 4 weeks and a maximum of 8 weeks. The biologically active factor present in the extract induced antheridia in gametophytes of *Onoclea sensibilis* and *Thelypteris* sp. even though such gametophytes did not form any antheridia spontaneously.

A reliable and sensitive assay method is described which makes use of the *Onoclea* gametophyte for the detection of antheridium-inducing activity. With this system the extract of 7-week-old *Pteridium* prothalli was shown to induce antheridia to a dilution of 1:31,250, while liquid medium on which *Pteridium* prothalli had been growing for the same period of time was active to a dilution of 1:6,250 to 1:31,250.

The active factor was stable to boiling, readily soluble in water, but sparingly soluble in ether, as found by Döpp. In addition it was found to be stable to autoclaving for 15 minutes under 15 lbs. atmospheric pressure, to boiling for 10 minutes at pH 2, but labile to boiling for 10 minutes at pH 12. It dialyzed freely and was adsorbed on charcoal from which it could be eluted by methanol.

The hypothesis that the extract induced antheridia by way of unspecific growth inhibition was refuted by the demonstration that the active factor induced antheridia without at the same time inhibiting growth and by the demonstration of its specific association with antheridium formation (extracts from the gametophytes of 2 ferns that form antheridia under the prevailing conditions of culture were 3,000 to 15,000 times more active than extracts from 2 ferns that do not form antheridia under these same conditions of culture). Thus the results reported here indicate that the biologically active factor is a natural metabolite specifically concerned with the induction of antheridia.

Preliminary investigations suggest that the active factor is present, and functional in antheridium formation, in all polypodiaceous ferns.

Acknowledgement

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DIFFERENTIAL GROWTH OF THE RAT SKULL

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INTRODUCTION

The growth of the rat skull has recently been reinvestigated (Baer, '54; Moss, '54a). Both of these studies established that simultaneous changes in absolute size and relative proportions occur with increasing age. Analysis of our combined data was undertaken to determine if this process of disproportionate growth could be expressed in a simple quantitative fashion.

Differential growth analysis aids in this task (Moss, '54b). It does so in the following way. Each cranial dimension increases at a specific growth rate which is constant for a greater or lesser period of time. These rates may, and often do, differ from each other. A constant ratio between differing specific growth rates produces simultaneous changes in absolute size and in relative proportions. Graphic analysis of the relationship between any two specific growth rates is made using log-log paper. If the ratio between these specific growth rates is constant, a straight line is produced. A change in this linear slope indicates a change in the ratio. This implies that while the absolute size continues to increase, there will be a change in the relative proportions from those previously existing. These changes of slope, or interphases, are indicative of critical developmental stages. One may confidently look for histomorphological and quantitative events which correlate with these stages (Moss, Noback and Robertson, '55a, b; Noback and Moss, '56; Noback, '55).

This paper presents a differential growth analysis of the post-

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natal rat skull. The relationships between the specific growth rates of the various bones and the relationships of different dimensions within certain individual bones, are reported. The existence of critical developmental stages, indicated by interphases, are noted. These are correlated with the development of related structures of the head. Specific attention is given to four problems: 1) the existence of growth gradients in the dorsal skull surface; 2) the existence of growth gradients in the basal skull surfaces; 3) the morphogenesis of individual bones; and 4) sexual differentiation.

Materials and Methods

The data represent measurements of 374 rats. Of these, 161 reported on by Moss ('54a), were unsexed below 34 days of age and grouped by age intervals. Baer ('54) reported on 77 rats, 50 males and 27 females, ranging from 1 to 101 days of age. The remaining 109 animals comprise a series made available by Dr. Maury Massler to one of us (MJB) who took the measurements reported in this paper. This series included 67 males ranging from 1 to 222 days of

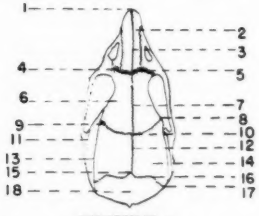

FIGURE 1
Anatomy of the calvaria of 34 day rat. Drawn from a tracing.
1—Anterior Nasal Point
2—Nasal Bone
3—Nasal Suture
4—Fronto-Nasal Suture
5—Nasion
6—Frontal Bone
7—Metopic Suture
8—Pterion
9—Coronal Suture
10—Bregma
11—Junction of Squama and root of Zygomatic Process
12—Sagittal Suture
13—Temporal Crest
14—Parietal Bone
15—Lambda
16—Anterior Lambdoid Suture
17—Asterion
18—Interparietal Bone

age; 37 females ranging from 30 to 280 days of age, and 5 unsexed animals 15 days of age.

The sources of these animals differed. One set of data represented a "Long-Evans Strain" (MLM); another set represented "pure strain albino rats" (MJB), while the remainder were albinos of a "modified Wistar strain." The effect the divergent sources had on the results will be commented on below.

The method measurement is shown in Figs. (1, 2, 3). All of our

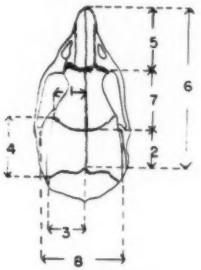

FIGURE 2

Dimensions measured indicated on a tracing of skull of 34 day control rat.

1—Pterion-Bregma
2—Bregma-Lambda
3—Lambda-Asterion
4—Asterion-Pterion
5—Nasal Length
6—Nasal Lambda
7—Projective Frontal Length
8—Squamosal width of skull

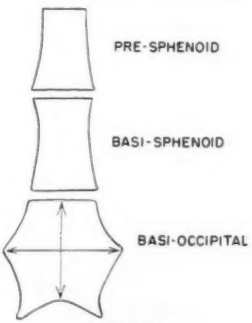

FIGURE 3

Diagrammatic representation of the skull base of the rats. The 3 bones measured are shown. The arrow indicates that in each case the length measurements were taken in the midsagittal plane and breadth measurements at the widest part of the bone.

measurements fell into two types: 1) measurements of the greatest lengths and the greatest widths of individual bones, and 2) measurements of skull diameters consisting of a series of adjacent bones. In the case of the parietal bone, all four borders of the bone were measured and all of these measurements are either duplications or variations of those of Hatai ('07).

The values were plotted on log-log paper in the following fashion. For any given combination of dimensions, a plot was made of the individual values of the material of Baer ('54) and of Massler. Separate plots were made for male and female data. These graphs were compared with those made from the data of Moss ('54a) which utilized the mean values of age groups. The existence of linear slopes was determined by inspection. The usual methods of determining regression lines were not utilized, since neither parameter may be considered as independent (see Waterman, '54). The angular value of the slope was determined. The tangent of this angle is the numerical value for the constant (k), in the differential growth equation

(1) $y = bx^k$, which may be rendered

(2) $\log y = \log b + k \log x$, where x and y are the

two diameters and where b may be considered to be only the "y intercept" of the slope.

This value of (k), then, gives the ratio between the specific growth rates of the dimensions x and y. When this value was 1.00, the slope of the line was 45° and the specific growth rates of x (the abscissal value) and of y (the ordinate value) were equal. When the value of (k) was less than 1.00, the slope of the line was less than 45° and the ratio favored the abscissal value (x); when the value of (k) was greater than 1.00, the slope of the line was greater than 45° and the ratio favored the ordinate value (y).

Results

The absolute mean dimensional values for 161 animals, up to 34 days of age, are shown in table (1). The tables of individual dimensional values for the remaining 193 animals are too lengthy for publication. They may be obtained from the authors.

Growth gradients; dorsal skull surface length. Combination of midsagittal lengths of the components of the dorsal skull surface are

plotted. These are, in antero-posterior order, the length of the internasal suture, the metopic suture, the sagittal suture and of the interparietal bone.

TABLE 1
Growth of the Normal Rat Calvaria

Age (days)	Measurement (1) Pterion-Bregma	Measurement (2) Bregma-Lambda	Measurement (3) Lambda-Asterion	Measurement (4) Asterion-Pterion	Number
C-N*	3.1±.03***	2.7±.11	2.2±.12	3.5±.03	16
1	4.5±.07	3.4±.13	3.5±.09	4.7±.07	11
2	5.3±.11	3.6±.05	3.8±.07	5.2±.07	22
3	5.3±.09	4.1±.07	4.7±.12	6.1±.08	15
4	5.4±.07	3.8±.08	4.5±.09	6.2±.12	10
7	5.5±.12	4.6±.12	5.2±.14	7.4±.26	12
10	5.9±.08	5.0±.15	5.8±.13	8.7±.17	19
15	6.2±.07	5.9±.11	6.2±.16	10.3±.18	16
20	6.1±.06	5.9±.05	5.8±.10	10.2±.15	12
30	6.4±.09	6.4±.10	6.6±.09	11.0±.08	10
34-R**	6.1±.05	6.1±.09	6.5±.07	11.8±.08	18
34-L**	6.1±.05		6.4±.07	11.3±.11	17

Age (days)	Measurement (5) Nasal-Length	Measurement (6) Nasal-Lambda	Measurement (7) Projective Frontal Length	Measurement (8) Squamosal Width	Number
C-N	2.7±.08	10.0±.21	4.6±.09	7.4±.12	16
1	3.5±.15	12.2±.06	5.3±.16	8.7±.14	11
2	4.2±.09	13.0±.13	5.4±.08	9.4±.10	22
3	4.7±.10	14.4±.18	5.6±.12	10.1±.12	15
4	4.5±.13	14.1±.13	5.8±.17	10.1±.16	10
7	5.3±.12	17.7±.28	7.7±.09	12.2±.36	12
10	6.3±.13	18.8±.28	7.4±.15	12.7±.17	19
15	8.4±.23	23.4±.26	9.2±.14	13.6±.26	16
20	8.9±.21	23.9±.24	9.0±.13	13.5±.14	12
30	11.2±.24	27.3±.12	9.8±.21	14.0±.09	10
34	11.5±.11	28.2±.23	10.6±.13	14.3±.08	18

* = Circumnatal age (within 24 hours of anticipated birth).
** = Right and left respectively.
*** = All values are the Mean values ± the Standard Error of the Mean.

The results are shown in table 2. It is seen that two such combinations do not show a change in linear slope, termed an interphase, while all of the others do (figs. 4, 5). A growth gradient exists in the dorsal skull surface which exhibits two phases. During the first phase, the descending order of specific growth rates is as follows: nasal, parietal, interparietal, frontal bones; while in the second phase the descending order of specific growth rates is nasal, frontal, parietal and interparietal bones. Although *identical* gradients are shown

TABLE 2
Antero-posterior growth gradients of the dorsal skull surface of the rat.

Ordinate (y)	Abscissa (x)	k_1	k_2
Length of Sagittal Suture (Parietal Bone)	Length of Metopic Suture (Frontal Bone)	1.54	.78
Length of Interparietal Bone	Length of Metopic Suture	1.28	.34
Length of Interparietal Bone	Length of Sagittal Suture	.82	—
Length of Metopic Suture	Length of Internasal Suture	.53	—
Length of Interparietal Bone	Length of Internasal Suture	.78	.29
Length of Sagittal Suture	Length of Internasal Suture	.90	.45

k is the constant in the differential growth equation

$$y = bx^k$$

and the subscripts 1 and 2 refer to values before and after the interphase (change in linear slope). All values taken in the midsagittal plane.

by all three strains of rats, the age at which the shift or interphase occurs varies with the strain. The shift was found to occur at the tenth day for the strain reported by Moss and at the fifteenth day for the strains used by Massler and by Baer. No sexual differences were noted or expected (Hatai, '07).

Growth gradients; basal skull surface length. Midsagittal measurements of presphenoid, basisphenoid and basioccipital bone lengths

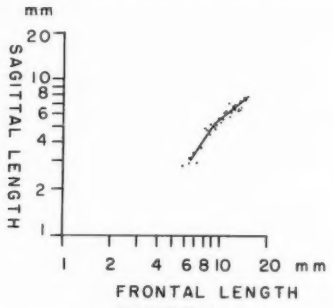

FIGURE 4

Ratio of the specific growth rates of sagittal suture length and frontal bone (metopic suture) length shown graphically. Observe the interphase. In figures 4 through 8 the lines are drawn from the combined mean data of Moss and the individual male data of Baer. The dots are selected from Baer's data and indicate the dispersion about the lines.

show a shifting growth gradient in the skull base. Presphenoid and basisphenoid bone lengths show identical specific growth rates ($k = 1.00$). These dimensions, as ordinates, are plotted against basioccipital length, as the abscissa. The following results are obtained. At first, both sphenoid bone lengths grow relatively faster than the basioccipital length ($k = 1.5$). A change in linear slope occurs, however, after which the ratio favors the growth of the basioccipital length ($k = .67$) (fig. 6). The present analysis suggests that this interphase occurs at approximately the same age as that found on the dorsal skull surface, although data for the base are not as abundant as for the dorsal surface.

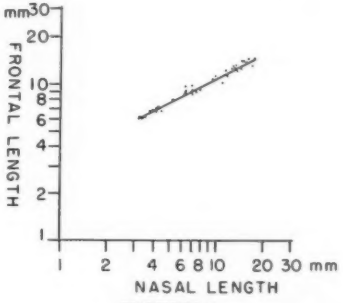

FIGURE 5

Observe the constant ratio of these two dimensions. A similar plotting was obtained for the ratio of the specific growth rates of the sagittal suture and the length of the interparietal bone. The dots on this graph are from male data of Baer. The slope of the line on this graph, as in all other graphs of male data, is capable of exact duplication with female data.

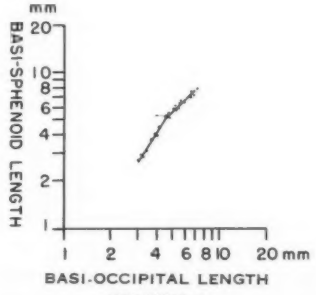

FIGURE 6

This graph indicates that after the interphase the ratio of these two specific growth rates favored the abscissal value, basioccipital length. Male data.

Plotting the midsagittal dorsal surface lengths, as ordinates, against basioccipital or basisphenoid lengths, as abscissae, confirms the existence of the shifting dorsal growth gradient.

Growth gradients; basal surface width. Throughout the period of development studies in this paper, the specific growth rate of the maximum basioccipital bone width is constantly greater than that of the maximum sphenoid width (k = 1.6) (fig. 7).

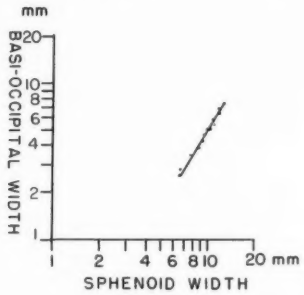

FIGURE 7

Note the lack of interphase in the ratio of the specific growth rates of these two dimensions. Male data.

Morphogenesis of bone form. Consideration of the ratios between the specific growth rates of the several *intraosseous* dimensions facilitates the analysis of form changes in the individual bones. Here, as in the skull as a whole, differences between the specific growth rates produce a simultaneous change in relative proportions with absolute increases in bone size.

In the case of the interparietal bone, for example, a constant ratio is observed between the greatest width, as ordinate, and the greatest length, as abscissa (k = .78) (fig. 8).

The morphogenesis of the parietal bone is somewhat more complicated. The bone is measured along its four sutures: coronal, sagittal, anterior lambdoid and temporoparietal. Figure 9 graphically illustrates the relationships between these four dimensions. The definitive morphology of the parietal bone is attained by the disproportionate growth of these diameters. A constant ratio is found between their specific growth rates. The numerical values (k) expressive of these ratios change in some cases. The lateral antero-posterior dimension grows relatively faster than the medial. Initially, the growth

in width predominates, but after the interphase, the length growth is faster. This results in a great morphological change between the infantile and adult bone. The age at which these interphases occur is the tenth day, in part of the material (MLM), and the fifteenth

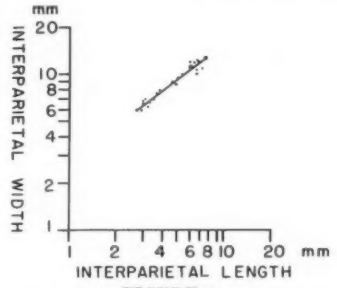

FIGURE 8

This *intraosseous* graph indicates that the length of the interparietal bone constantly increases relatively faster than the width, thus accounting for the morphological changes observed with age. Male data.

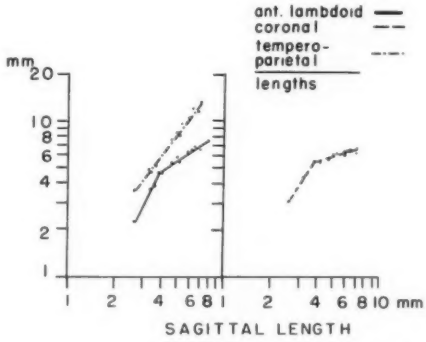

FIGURE 9

As in figure 5, this intraosseous graph gives a simple quantitative expression to the changing shape of the parietal bone with age. Dots represent mean values for age groups reported in table (1).

day in the remaining material (MJB, MM). This strain difference in material has been noted above.

Sexual differentiation. Sexual differences in skull morphology at a given age have been noted in the adult rat by Hatai ('07), and in the growing rat by Massler and Schour ('51). This may be due to

either one of two reasons. First, there may exist sexual differences in the ratios between the specific growth rates of a given pair of dimensions. Second, the *ratio* between the specific growth rates in both sexes may be identical, but the *specific growth rates* of each of the pair of dimensions may be changed.

Our data support the second hypothesis. Plots of male dimensions do not differ in slope from those of female dimensions. The absolute female dimensions appear, in general, to be smaller than the male at any given age.

Discussion

Growth gradients. The existence of several coordinated patterns of differential growth in the rat skull is demonstrated. It is important to notice that this statement implies nothing concerning the processes by which this growth occurs. This topic is properly approached by gross and microscopic observations. Descriptions of these processes are given in detail by Massler and Schour ('51), by Baer ('54) and by Moss ('54).

In essence, we are stating that whatever these processes are, and however they function, they do so in a coordinated fashion which is capable of simple quantitative expression by the differential growth formula: $y = bx^k$. The constant (k) gives an easily computed value which expresses the relationship between the specific growth rates of the various skull dimensions.

The growth of the rat skull from natal to adult form entails a change in relative proportions as well as in absolute size. The existence of a differential growth relationship between gross segments of the skull (vault length, face length and base length) has previously been reported (Baer, '54). That paper confirms, in the rat, the existence of similar relationships observed in mice (Green, '33).

The present paper goes on to show that within these overall cranial growth patterns a regressus of additional growth patterns are found. These are descriptive of the unitary osseous dimensions of the skull. The pattern of growth in skull length reveals a critical phase of changing relationships between specific growth rates. The implications of this interphase will be discussed in the next section.

Correlated Histomorphological changes. It is commonly observed that in the rodent crania, following the general mammalian pattern, the neural skeleton matures more rapidly than the facial skeleton.

We are now in a position to make rather precise statements concerning this event. We note a shift in the gradient of dorsal skull growth at an early age (10-15 days). Prior to this shift, or interphase, the parietal and interparietal bones were growing relatively faster in length, than the frontal bones. This order is reversed after the interphase. The occurrence of this interphase is clearly indicated by our data. Let us investigate the growth of related structures of the head at this critical developmental horizon. What gross and microscopic changes correlate with this interphase?

When body weight is plotted against brain weight for the rat, an early rapid increase of brain weight is seen which shows a definite interphase. A marked slowing of the specific growth rate of the brain follows. This interphase occurs at the identical age observed for the interphase in the length gradients of the skull (fig. 10), (Sugita, '17; Asling, et al., '52; Walker, et al., '52).

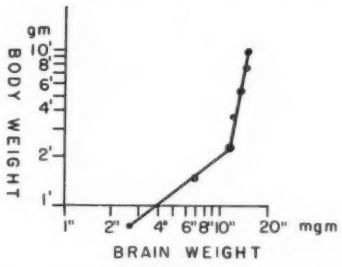

FIGURE 10

The data points are derived from the papers of Asling et al. ('52) and of Walker et al. ('52), both of whom reported on identical rats. Note the clearly indicated interphase.

On a microscopic level, the age of the cranial and the brain weight interphases, in the rat, are marked by other events. The growth of the cerebral cortical thickness also shows an interphase at the same age. The earlier period is marked by rapid cellular immigration, cell multiplication and by cell enlargement. These processes either cease or slow markedly after this interphase (Sugita, '17).

In summary, the shifting of the dorsal cranial growth gradient in favor of the facial skeleton growth is not an isolated event. It is well correlated with changes in the underlying neural mass.

Strain Differences. Reference has been made in this paper to the differences in the ages at which the several strains studied showed the observed interphases. This discrepancy is not as critical as it might first appear. Such factors as litter size, birth weight, intrinsic growth rate, nutritional status, individual, sexual and genetic differences must be considered (Green, '31a, b; Outhouse and Mendel, '33). From the evidence available we may conclude that age is not a critical determinant of bodily proportions. The different ages at which the interphases were observed may reflect some or all of the above mentioned factors. The effect of nutritional status and of strain differences on brain growth is most strikingly shown by comparison of the data of Sugita ('17) with those of Asling et al. ('52). The Long-Evans strain of the latter authors shows consistently higher brain weights at all given post-natal ages.

The point to be stressed, however, is that *despite* these strain differences as to age at which interphases occur, identical growth gradients and identical osseous dimensional relationships were found. Corroborative evidence for this statement is given by MacArthur and Chaisson '45, p. 309) who state that "mice all form one allometric tribe, not differing in (b) or (k) constants."

It is exactly the properties of differential growth analysis which allows us to compare such disparate data. In such an analysis we are able to abstract from morphology, from factors of nutrition, from absolute values of magnitude, and most important of all, from time (Needham, '50).

Sexual differentiation. Massler and Schour raise the question of whether the observed differences between male and female rat skulls are ". . . the result of a steeper gradient of growth, a smaller growth potential, or both . . ." (Massler and Schour, '51). If we understand their term "gradient of growth" as referring to the constant (k), we may now state that sexual differences are not due to differences in this constant. Continuing to utilize their terminology, we may say that the female rat has a "smaller growth potential" than the male.

Conclusions

The growth of the rat skull during the first 280 days of life was studied by differential growth analysis and the description of the changing proportional relationships was reduced to simplified quantitative expression.

The growth gradients of the skull are demonstrated. The neural skull, at first, grows relatively faster than the facial skull. A reversal of gradient occurs in both dorsal and basal skull surfaces. On the dorsal surface, this shifting favors the growth of the facial skeleton. The age at which this shift, or interphase, occurs is found to exhibit strain differences.

Correlative histomorphological changes in the brain are described. The changes occur at the same age at which the skeletal growth gradients shift.

Sexual differentiation in the skull is found to be due to differences in growth potential rather than to differences in proportionality of parts.

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OOGENESIS IN ADULT *DROSOPHILA MELANOGASTER*[1]

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INTRODUCTION

The general morphology of the ovary of the adult fruit fly, *Drosophila melanogaster*, has been described by Miller ('50), and this paper represents an extension of his work. We have subdivided the development of the egg into various successive stages to provide a foundation for later work. In the future we intend to study the distribution of these stages in the ovary as affected by age and other physiological conditions. Among the criteria used in characterizing stages are: (1) the size and location of the cysts or egg chambers, (2) the size of the oocyte relative to the nurse cells or to the entire egg chamber, (3) the cytology of the oocyte and nurse cells, (4) the ratio of cytoplasm to nucleoplasm in the nurse cells, (5) the morphological classes of nurse nuclei in the egg chamber, (6) the presence or absence of border cells, (7) the height of the follicular epithelium, and (8) the degree of development of such structures as the wall which separates the nurse cells from the oocyte, the chorion, the micropyle, and the dorsal appendages.

MATERIAL AND METHODS

Female *Drosophila melanogaster* of various ages belonging to the Oregon-R wild type strain were used in this study. All flies were raised at 25° C. The ovaries were dissected from etherized flies immersed in *Drosophila* Ringer solution (NaCl, 0.17M; KCl, 0.005M; $CaCl_2$, 0.002M) containing the wetting agent, Tergitol (0.05%). The ovaries were fixed in Kahle's fluid and stained according to the Feulgen procedure as modified by Whiting ('50). Ovaries fixed in Carnoy (6:3:1) and stained in Feulgen (hydrolysis 15 min.) gave preparations very similar to those fixed in Kahle's fluid. The majority of

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the study is based on Kahle-Feulgen whole mounts. However, whole mounts freeze-dehydrated according to the method of Woods and Pollister ('55) were also used. These were mounted in mineral oil and examined under phase contrast. Material fixed in Baker's fluid (Baker, '50) and stained according to the Feulgen method was especially useful for studying the germarium. A relatively small amount of material was fixed in aqueous Bouin, sectioned at 7μ and stained with iron haematoxylin or toluidine blue. Neutral formalin and buffered OsO_4 proved unsatisfactory for fixation of this material.

Observations were made in normal bright field, phase contrast or polarized light utilizing a Zeiss Opton, stand W research microscope equipped with optovar, photochanger and a number 4, revolving bright field-phase contrast condenser system. A 4 x 5, American optical, fixed length (250 mm) camera (minus beam splitter and shutter) was mounted above the photochanger. The N.A. of the condenser was held to ¾ the N.A. of the objective used in all bright field photographs. For low and medium magnifications in bright field, Zeiss planachromats were used at their corrected wave length of 550 mμ. High power photographs were made with a 100x, oil immersion, apochromatic objective. An 8x compensating ocular was used throughout the entire series of photographs. The Farrand interference filter (550 mμ) remained in the system with phase contrast optics. The choice of sheet film (either Kodak contrast process ortho or panatomic X) varied according to the degree of contrast in the specimen, the magnification and the nature of the object to be resolved.

The larger chambers (stages 8-14) in Kahle-Feulgen whole mounts have undergone considerable shrinkage, but this did not occur in freeze-dehydrated preparations. Measurements utilizing an ocular micrometer were made of ovaries prepared by the Kahle-Feulgen and the freeze-dehydration techniques. It was possible to arrive at shrinkage factors from discrepancies between measurements made of the two types of preparations. All measurements given are corrected for shrinkage.

Stages of Oogenesis

The two ovaries of the Oregon R, wild type strain of *Drosophila melanogaster* are each subdivided into an average of 12 egg tubes or ovarioles. The ovariole is differentiated into an anterior germarium

and a series of egg chambers. The germarium (Figures 3-6) is generally 20 x 60 micra ($v = 1.3 \times 10^4 \mu^3$) and contains up to 200 cells, each of which is about 5μ in diameter. At the apex of the germarium is a region containing 50 or so mitotically active cells. It is assumed that a germarial cell divides into two daughter cells. One of these cells repeats the process, while the other oogonium undergoes four consecutive, synchronous divisions (Figure 52) to produce a cyst of 16 daughter cells. These divisions occur in a series of transversely arranged, lens-shaped, incipient cysts. The 16 daughter cells eventually form a spherical cyst, the germarial cyst, in the posterior region of the germarium. It is incompletely covered by a layer of follicle cells and is often separated from the closest incipient cyst by an indentation in the germarium and by a transverse layer of follicle cells. The nuclei of follicle cells are ellipsoidal (1.5 x 3μ), whereas the 16 cells comprising a cyst have spherical nuclei (4-5μ in diameter). In the germarial cyst it is sometimes impossible to distinguish the daughter cells from one another, but more often the most posterior one has more condensed and deeply staining chromatin. It is this cell which becomes the oocyte. Occasionally there appears in the germarial cyst a second cell which is adjacent and similar in morphology to the oocyte. Perhaps this is the "trophocyte" of Guyenot and Naville ('33). However, in the very next stage this cell is indistinguishable from the other 14 nurse cells. Unfortunately, we are unable to say anything about the cytology of any of these nuclei because of their small size. Once the cyst has been completely surrounded by a layer of follicle cells, it is pinched off from the germarium and so becomes the first egg chamber.

In our terminology oogenesis is subdivided into 14 stages with the germarial cyst and the first chamber as stages 1 (Figure 4-7 and 22) and 2 (Figures 8-10 and 22), respectively. The 15 nurse cells at stage 2 contain pale-staining, thread-like chromosomes, but in stage 3 the chromosomes which meander through the nucleoplasm are thicker and more deeply stained (Figures 11-13 and 22). Stage 3 oocyte nuclei are the first to show a Feulgen-positive nucleolus or karyosome. At stage 4 (Figures 14-16) the nurse cell nuclei contain several Feulgen-positive masses, some of which are connected together by fine strands. Stage 5 chambers (Figures 17 and 18) are characterized by a nonhomogeneous group of nurse cell nuclei some of which look like those of stage 4 and some like those of stage 6. The

chromatin of nurse cell nuclei belonging to stage 6 (Figures 19-21) stains densely and looks spongy and amorphous. The stage 6 egg chamber is spherical, but the stage 7 (Figures 22, 33 and 34) chamber is the first of the larger, ellipsoidal chambers. Here the nurse cells and the oocyte are more or less equal in size, but stage 8 (Figures 22, 35 and 36) is characterized by an oocyte which is significantly larger than the average nurse cell. At stage 9 (Figures 23, 38, 39 and 40) the oocyte makes up ¼ to ½ the total volume of the chamber, and the border cells make their appearance. Border cells (cf. page 144) are also present in stage 10 chambers (Figures 24 and 41). The oocyte makes up more than ½ but less than ¾ the volume of the stage 10 chamber. Stage 11 (Figure 25) chambers usually lack border cells. The oocyte makes up about ¾ of the total volume of the chamber, and an epithelial wall completely separates the oocyte from the nurse cells for the first time. The oocyte has almost reached its maximum size in the stage 12 chamber (Figures 26 and 42) and the densely-staining nurse nuclei are present as an anterior cap. These nuclei start to degenerate at stage 13 (Figures 27 and 29-31). The micropyle is completed, but the dorsal appendages are only partly grown. Stage 14 is the fully grown, ovarial, primary oocyte (Figures 28, 32 and 43) with completed dorsal appendages.

Stage Distribution as a Function of Age

This study is still in progress, but certain generalizations can be made at this time. Female flies which are between 0 and 1 hours old have 6-7 developing eggs per ovariole. Stage 7 is the latest found. Stage 5 makes up 10% of the total; the others 13-17%. These findings are in agreement with Bergner ('28). Virgin, 24-25 hour old, females, show 8-9 developing eggs per ovariole. All stages are found but stages 1-7 make up 85% of the total. The posterior chamber of any ovariole may belong to any one of 7 stages. Stage 8 occurs about 6%, stages 9, 10, 12, 13 and 14 each 1-2% and stage 11 less than 1% of the time. Females belonging to the following age groups were also studied: (1) Virgins—96-97 hours old, (2) 240-241 hour old females which were allowed to mate from the 24th hour on, (3) 240-241 hour old females which were allowed to mate from the 96th hour on, (4) 504-505 hour old females which were allowed to mate from the 24th hour on, and (5) 504-505 hour old females which were allowed to mate from the 96th hour on. The ovaries from the flies belonging to these five series were similar

in morphology. They contained 7-8 developing eggs per ovariole plus a stage 14 oocyte. The egg chamber immediately behind the mature egg is always in stage 7. Oregon-R females under optimal conditions during their period of maximum egg production can lay 70 eggs or 3 eggs from each tube daily. It follows that an egg can develop from stage 7 to 14 (i.e. increase in volume by a factor of 43 times) in a period of 12 hours or less.

These data are of some use to those studying the radiation genetics of the *Drosophila melanogaster* female. It is obvious that both the distribution of chambers among various stages and the number of chambers in the ovaries is very different in freshly hatched females and more mature females (4 days old). Freshly hatched females have 150 developing eggs distributed among stages 1-7. Four day old females have 180 egg chambers distributed among stages 1-7 and 14. Thus flies irradiated and mated at an age of 4 days would produce about 24 eggs which were mature when treated and subsequently eggs which were in the same stage at treatment as the most mature chambers of female flies treated when freshly hatched.

Development of the Egg Chamber

During oogenesis the spherical germarial cyst undergoes a 5000-fold increase in volume and develops into an elongate mature egg (Figure 1). Egg chambers at stages 2 and 3 are ellipsoidal; those at stages 4, 5 and 6 ellipsoidal to spherical. The major amount of elongation occurs during stages 7-9 when the ratio of length to width increases from 1.5 to 2.4. The ratio of length to width is 2.5-2.6 for stages 10-14. The dimensions (in μ) of chambers characteristic of various stages are as follows: stage 1, 15 x 15; stage 2, 20 x 30; stage 3, 25 x 40; stage 4, 38 x 48; stage 5, 50 x 65; stage 6, 65 x 70; stage 7, 65 x 100; stage 8, 90 x 135; stage 9, 120 x 285; stage 10, 140 x 360; stage 11, 160 x 410; stage 12, 185 x 460; stage 13, 190 x 500; and stage 14, 190 x 500. The volume can be calculated from the formula $v = 0.5236\ w^2 l$, where v = volume, w = maximum width, l = maximum length.

Development of the Oocyte

The oocyte undergoes a remarkable growth during oogenesis (Figure 1). Between stages 1 and 14 it increases in volume by a factor of over 100,000 times. Under optimum conditions it probably takes 3 days for the completion of this process. During the first seven

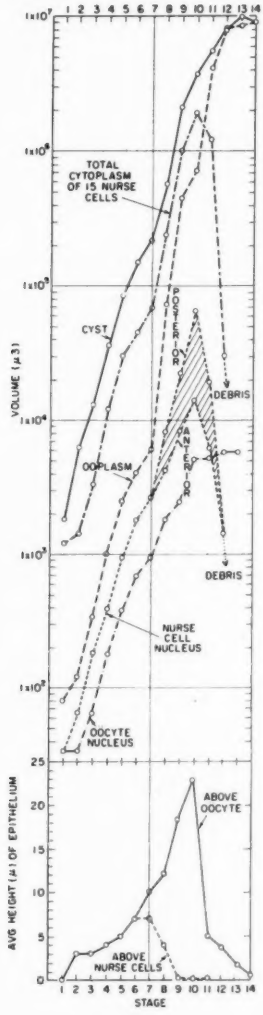


FIGURE 1

Change in volume or height as a function of stage during oogenesis in *Drosophila melanogaster*.

stages the oocyte and the nurse cells grow at identical rates, but subsequently yolk formation begins, and the oocyte grows faster. By stage 11 the oocyte contains more cytoplasm than do all the nurse cells put together, and by stage 12 the oocyte has almost reached its maximum volume (9×10^6 $\mu 3$). The oocyte and the nurse cells are separated from each other by a wall of follicle cells which begins to form late in stage 9. The wall is first observed at the periphery; it works its way inward and is completed during stage 11.

The oocyte nucleus increases in size from stages 2-10, and it subsequently remains fairly constant in volume until the nuclear membrane disappears late in stage 13 (Figure 1). In the first egg chamber it is always possible to distinguish the oocyte nucleus from the nurse cell nuclei, because it has condensed chromatin which stains more deeply than does nurse cell chromatin. The oocyte is always the posterior-most cell in the cyst. The spherical oocyte nucleus at stages 3 through 8 contains a characteristic, compact, Feulgen-positive nucleolus or karyosome (Figures 11, 19, 22 and 33-36). This body is 2 to 3μ in diameter. In these early stages fine strands are often seen associated with the karyosome (Figures 19, 33, 34 and 36). Such a karyosome has been observed in the oocyte nuclei of other *Drosophila* species (*virilis, pseudoobscura* and *gibberosa*). When viewed under phase contrast the oocyte nucleus of *D. virilis* shows in addition to the karyosome a Feulgen-negative plasmosome of similar size (Figure 37). In *melanogaster* plasmosomes ($d = 5\mu$) are often observed. During stages 9 and 10 the oocyte nucleus becomes elliptical, while the karyosome is less compact (Figures 23, 24, 39 and 41). It is generally located peripherally in the nucleus and has a diameter of 5μ. The nucleoplasm takes on a reticulate appearance. At stage 9 (Figure 39) a crescent-shaped aggregation of spheres is sometimes found in the nucleus, and this may represent an association of plasmosomes. During stages 11 through 13 (Figures 25, 26 and 42) the nucleus takes on a spherical shape and the nucleoplasm remains reticulate. The karyosome is compact (3μ in diameter) and is located centrally. Late in stage 13 the nuclear membrane disappears and all that remains is a Feulgen-positive body, the karyosphere (Figure 43), 2μ in diameter which is located in the anterior, dorsal portion of the egg.

The Development of the Nurse Cells

From stages 1 through 7 the nurse cells grow at a rate equal to the oocyte (Figure 1), and during this period the cells contain 1 to 2 times as much cytoplasm as nucleoplasm. From stages 8 through 10 the nurse cells continue growing, but are outdistanced by the volume increase of the oocyte. During this period the value describing the ratio of cytoplasm to nucleoplasm increases to 6. The nurse cells begin to shrink during stages 11 and 12 and are subsequently resorbed.

Nurse cell chromosomes are thin and pale-staining during stages 1 and 2 (Figures 8 and 9). They become readily visible as thicker, meandering threads associated with Feulgen positive blobs at stage 3 (Figures 11-13). Stage 4 nuclei contain densely staining bulbs (Figures 14-16) some of which are connected together by finer strands. Stage 5 chambers (Figures 17 and 18) contain nurse nuclei in varying degrees of maturity; some are similar in morphology to stage 4, others to stage 6. In the latter case the chromosomes fill the nucleus and give it an amorphous, spongy appearance. Such darkly staining nuclei are characteristic of stages 6 through 9 (Figures 19-22), and during this developmental period it is obvious that the DNA content per nucleus increases steadily. At stage 10 the nurse nucleus reaches its maximum size and has a volume approximately 2,000 times that of the stage 1 nucleus. The nuclei in a given cyst, however, are conspicuously nonhomogeneous with respect to size (Figures 1 and 2). Those nearest the surface of the oocyte are the largest and frequently have six times the volume of the anteriormost cells. This differentiation in nuclear size is less evident during stages 8 and 9. The nuclei of stage 10 nurse cells appear pale and contain chromosomes which are maximally extended and show lateral loops characteristic of the lampbrush condition (Figure 44). Painter mentioned the lampbrush-like structure of nurse cell chromosomes as early as 1940. The lateral loops often have a circumference of 12μ. Numerous spheres are located in the center of stage 10 nuclei (Figure 45), and these are also conspicuous in freeze-dehydrated preparations. These organelles are probably not artifacts, but plasmosomes (Hsu and Hansen, '53). Pale nuclei similar to those characterizing stage 10 chambers are often seen late in stage 9 (Figure 23) and early in stage 11. The nurse cell nuclei shrink during stages 11 and 12 (Figures 25 and 26) and subsequently degenerate (Figures

27, 29 and 30). They are resorbed except for some debris which is voided along with the egg. Between stages 10 and 12 the average nuclear volume decreases by a factor of 30 and the contents of the average nucleus is compressed. As a result these nuclei stain densely.

Development of the Follicle Cells and Associated Structures

The follicle cell layer is first complete in the stage 2 egg chamber. The height of the follicular epithelium increases from 3μ to 7μ during stages 2 through 6 (Figure 1). During stage 7 (before yolk

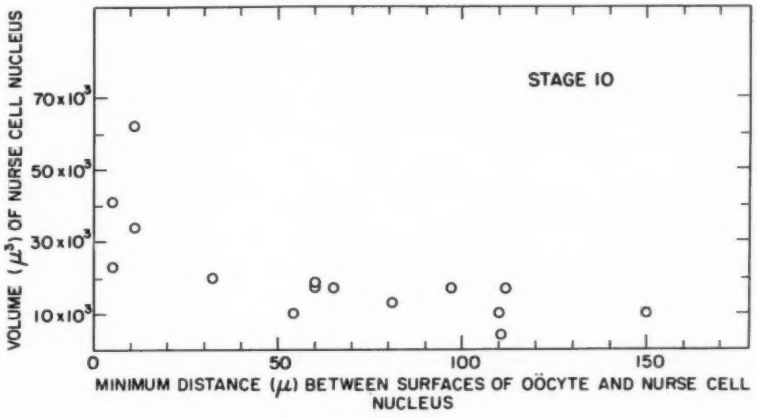


FIGURE 2
Nurse cell nuclear volume as a function of location in the stage 10 egg chamber.

begins to accumulate in the ooplasm) the follicular epithelium above the oocyte increase in height to 10μ. At stage 8 those follicle cells above the nurse cells begin to shrink. By stage 9 and thereafter they form a squamous epithelium less than 0.5μ thick. The epithelium above the oocyte continues to increase in height until stage 10 where a columnar epithelium 20-26μ high is produced. The nuclei of all follicle cells are centrally located. In freeze-dehydrated preparations those cells above the oocyte in stages 9 and 10 show numerous secretion vacuoles. Subsequent to stage 10 the columnar epithelium shortens abruptly and by stage 14 the epithelium is 0.5 to 1μ in height.

The chorion is a product of the follicle cells. It is laid down be-

FIGURE 3

The anterior region of an ovariole of *Drosophila melanogaster* showing the germarium and the first three egg chambers. Mitoses are seen in the germarium, while secretion vacuoles are observed in nurse cells in egg chambers 2 and 3.

FIGURES 4-6

Germaria showing lens-shaped or elliptical incipient cysts and a spherical germarial cyst. The germarial cyst is stage 1 in our terminology.

FIGURE 7

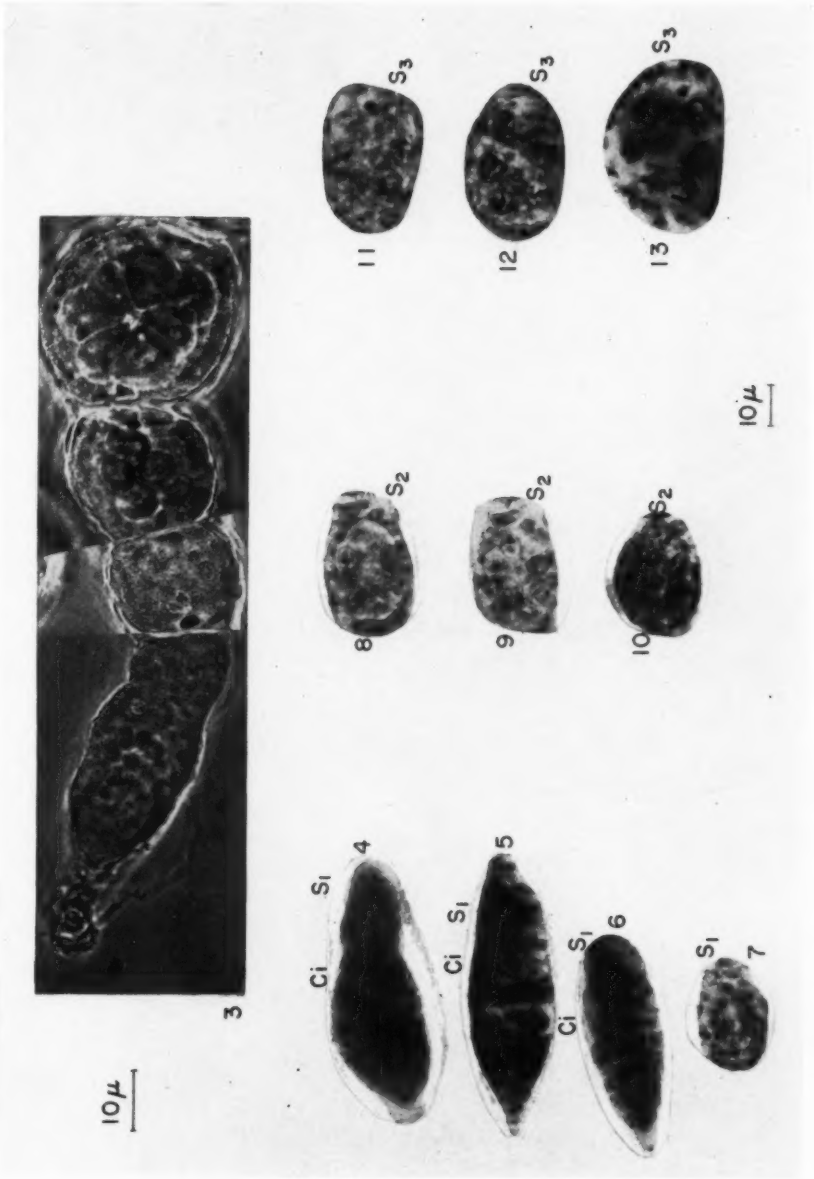
Stage 1. Germarial cyst showing an oocyte nucleus which is characterized by a u-shaped accumulation of condensed chromatin.

FIGURES 8-10

Stage 2 nurse cell nuclei are pale-staining; whereas the oocyte nucleus (Figures 9 and 10), located in the posterior periphery of the chamber, shows deeply stained, condensed chromatin.

FIGURES 11-13

Stage 3. Oocyte nucleus which is located in the posterior periphery of the chamber seems empty except for the characteristic karyosome (Figure 11). Nurse cell nuclei are partially filled by fine, meandering chromatin strands and larger blobs.


10μ
3
Ci
S1
4
Ci
S1
5
Ci
S1
6
S1
7
S2
8
S2
9
S2
10
S3
11
S3
12
S3
13
10μ

FIGURES 14-16

Stage 4. Nurse nuclei contain dense blobs of chromatin connected by finer strands.

FIGURES 17 AND 18

Nurse nuclei are morphologically nonhomogeneous. The anterior ones resemble stage 4; the posterior ones stage 6.

FIGURES 19-21

Stage 6. Nurse cells completely filled by chromosomal material. Karyosome and chromatic strands distinct in the enlarging nucleus of the oocyte (Figure 19).

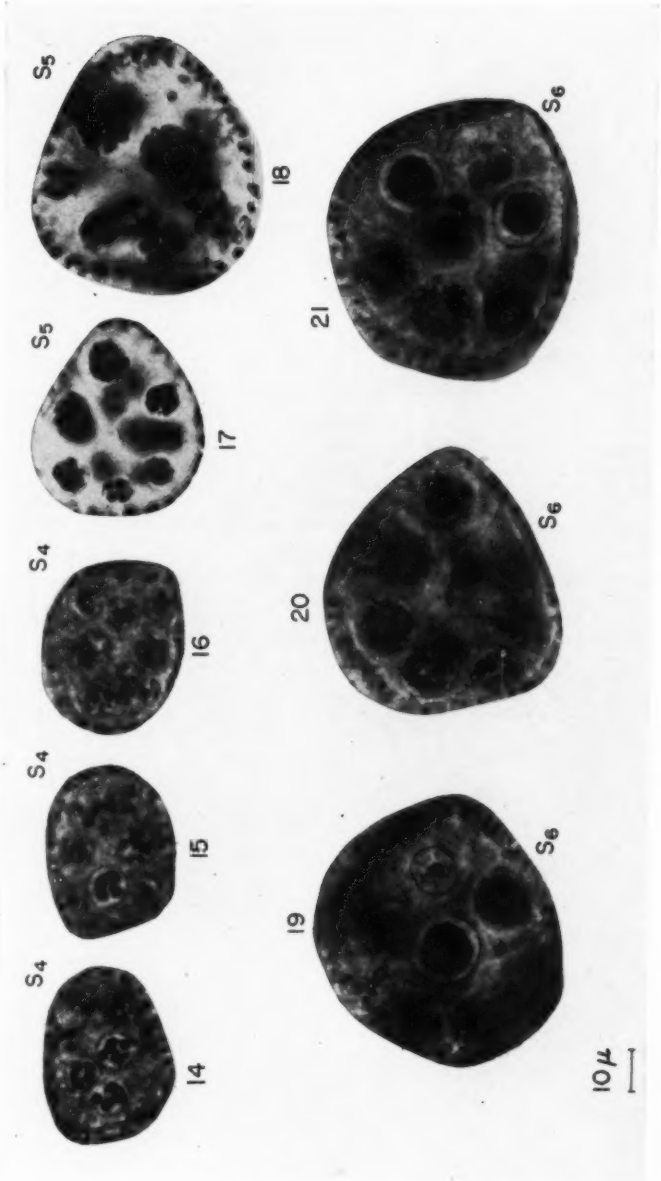

S4
14
S4
15
S4
16
S5
17
S5
18
S6
19
S6
20
S6
21
10μ

FIGURE 22

An ovariole of a 24 hour old *Drosophila melanogaster* showing the germarium and cysts in stages 1, 2, 3, 6, 7 and 8. Karyosomes are prominent in the nuclei of stage 3, 6, 7 and 8 oocytes. The stage 7 cysts are the first to show ellipsoidal shape. The stage 8 cyst shows the oocyte beginning to enlarge. The epithelium around the nurse cells is cuboidal while that surrounding the oocyte is becoming columnar.

FIGURE 23

Stage 9. The oocyte now makes up about 1/3 of the cyst volume. The nurse cell nuclei closest to the oocyte are somewhat larger than the rest. Nurse cell chromosomes are in the lampbrush condition. A squamous epithelium surrounds the nurse cells, while a columnar epithelium surrounds the oocyte.

FIGURE 24

Stage 10. The oocyte makes up about ½ of the cyst volume. The nurse cells bordering the oocyte now show distinctly larger nuclei. Nurse cell chromosomes are in the lampbrush condition. The follicular differentiation into squamous and columnar epithelium is very striking.

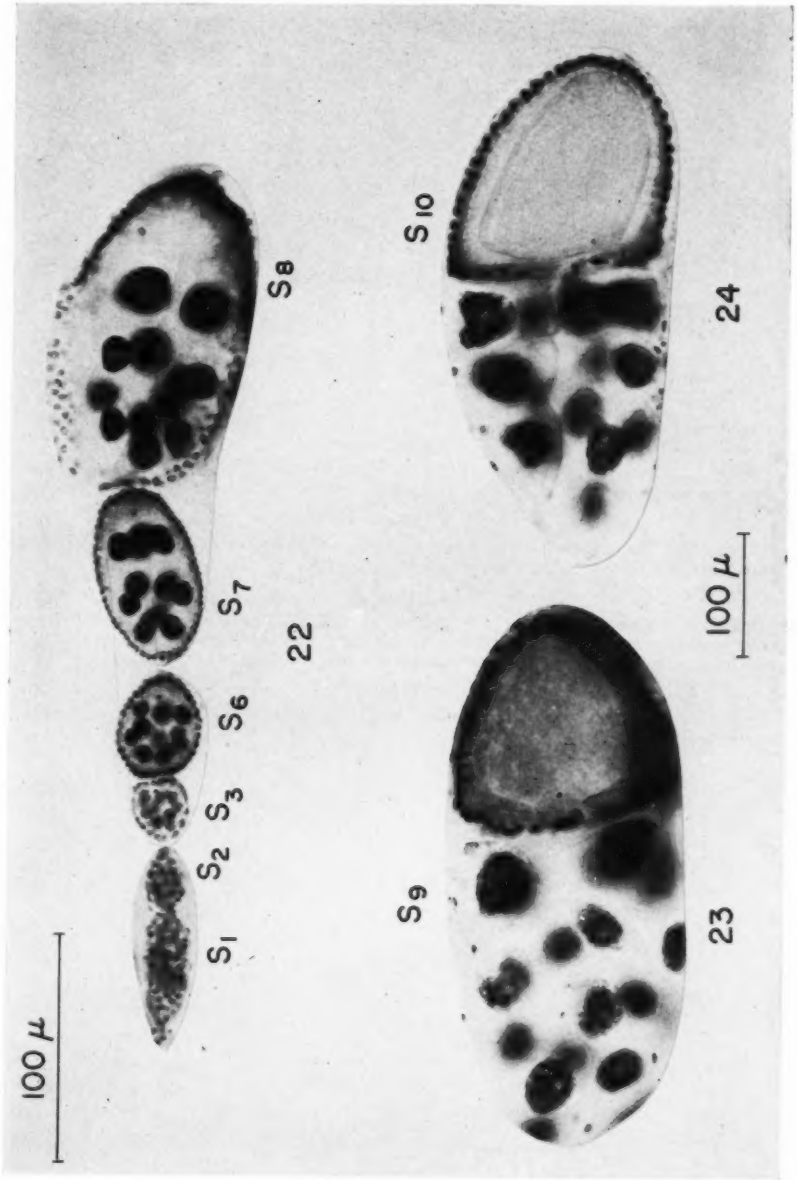

100 μ
S1
S2
S3
S6
S7
S8
22
S9
23
S10
24
100 μ

FIGURE 25

Stage 11. The oocyte makes up 2/3 of the cyst volume. The nurse cell nuclei have shrunken and stain homogeneously. The karyosome is still prominent.

FIGURE 26

Stage 12. The oocyte is nearly maximum size. The oocyte nucleus has reached maximum size. Nurse cell nuclei are crowded together at anterior pole.

FIGURE 27

Stage 13. Nurse cell nuclei are present as debris. The needle-like micropyle is clearly visible in the anterior region of the egg. The epithelium anterior to nurse cells is elongating to form characteristic appendages.

FIGURE 28

Stage 14. A dorsal view of a mature egg in an ovariole. Note the characteristic appendages. The follicular epithelium has been sloughed off one side of the egg.

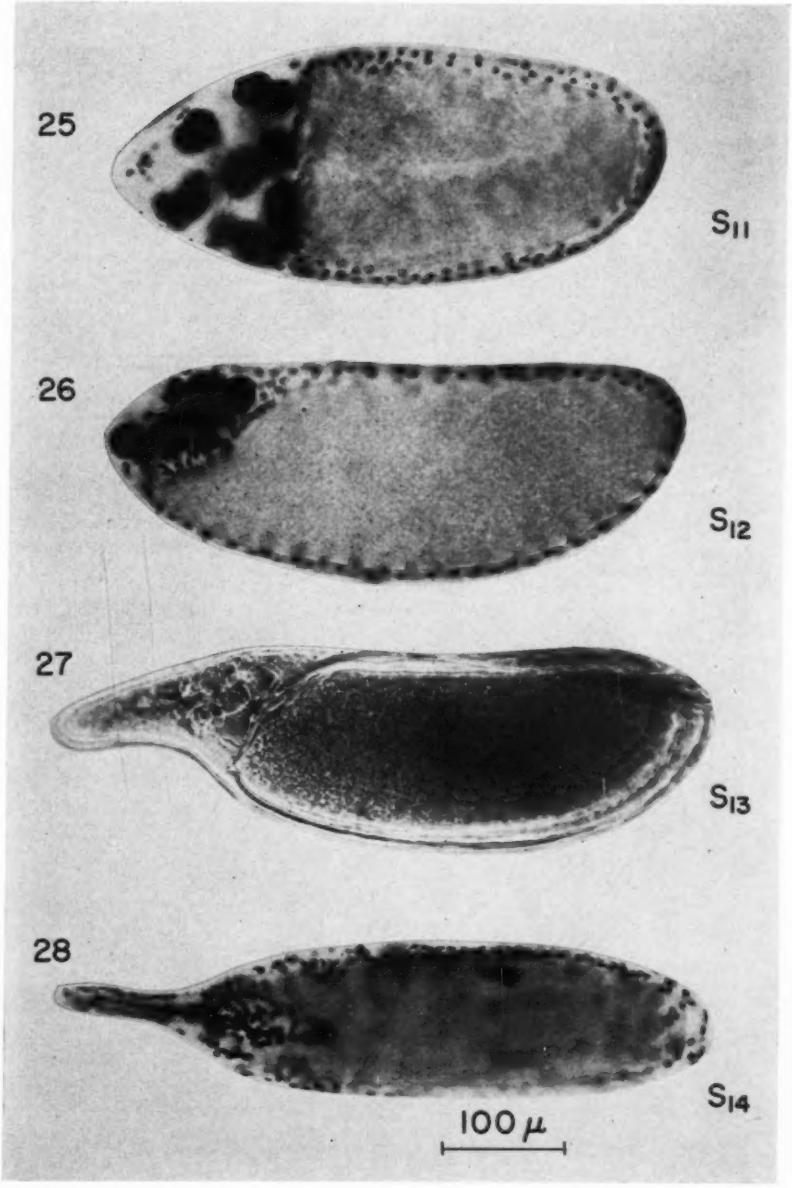

25
S11
26
S12
27
S13
28
S14
100 μ

FIGURE 29

Stage 13 oocytes showing developing micropyle (in the lower egg) and nurse cell debris.

FIGURE 30

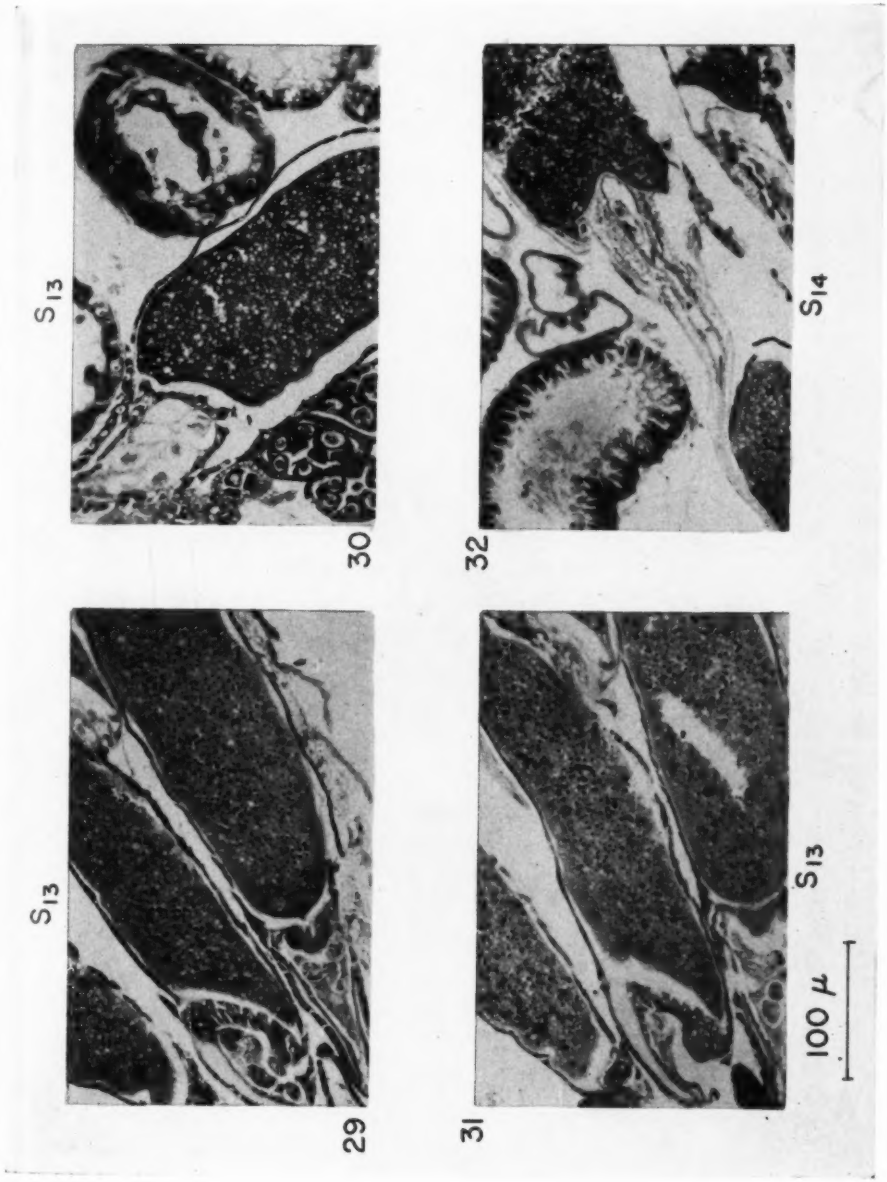
A stage 13 oocyte showing the developing filaments and the further degeneration of nurse cell nuclei.

FIGURE 31

A stage 13 oocyte showing a filament elongated to 1/3 the final length. The nurse cells are almost completely resorbed.

FIGURE 32

Stage 14, an oocyte located posteriorly in the ovariole of a 4 day old fly. The tips of the completed filaments are contiguous with the posterior portion of a more anterior egg.


S13
30
S13
29
S14
32
S13
31
100 μ

FIGURES 33 AND 34

Stage 7 chambers showing oocyte nucleus. Strands are observed associated with the karyosome.

FIGURES 35 AND 36

Stage 8 chambers showing oocyte nucleus.

FIGURE 37

Stage 8 chamber of *Drosophila virilis*. Here the oocyte contains a Feulgen-positive karyosome and a plasmosome which is clearly visible (phase contrast).

FIGURE 38-40

Stage 9 chambers showing border cells, oocyte nucleus and (Figure 40 only) chorion.

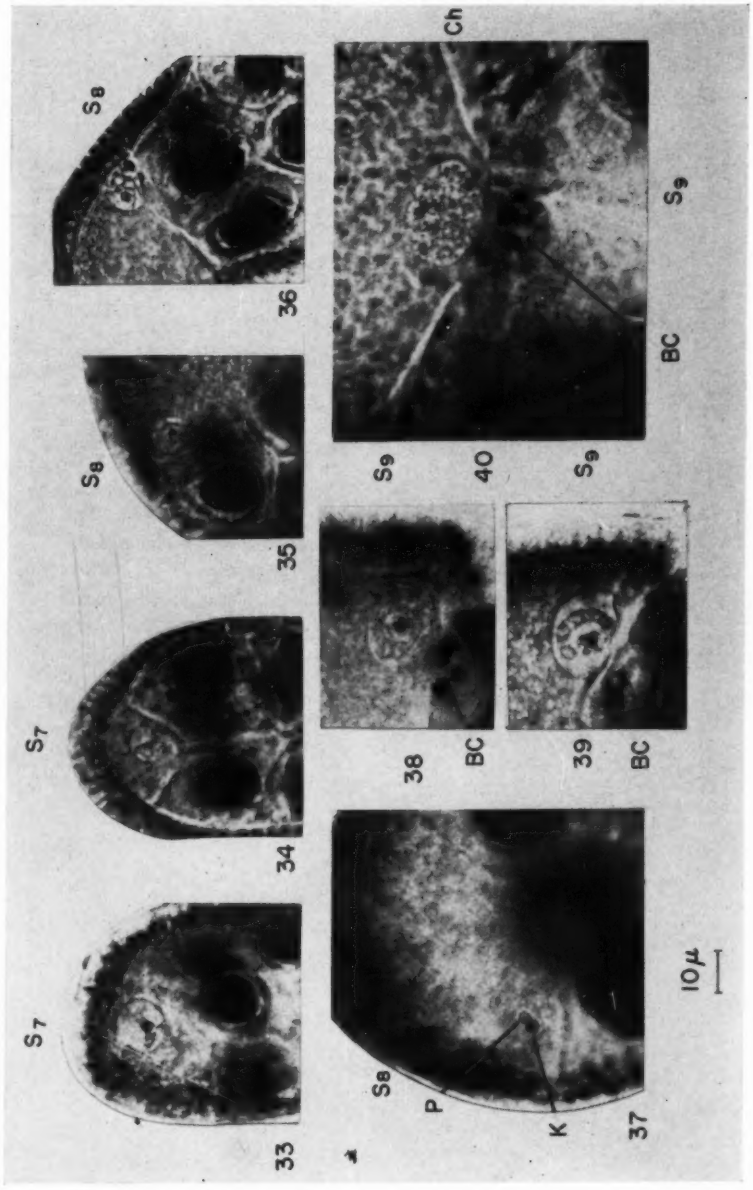
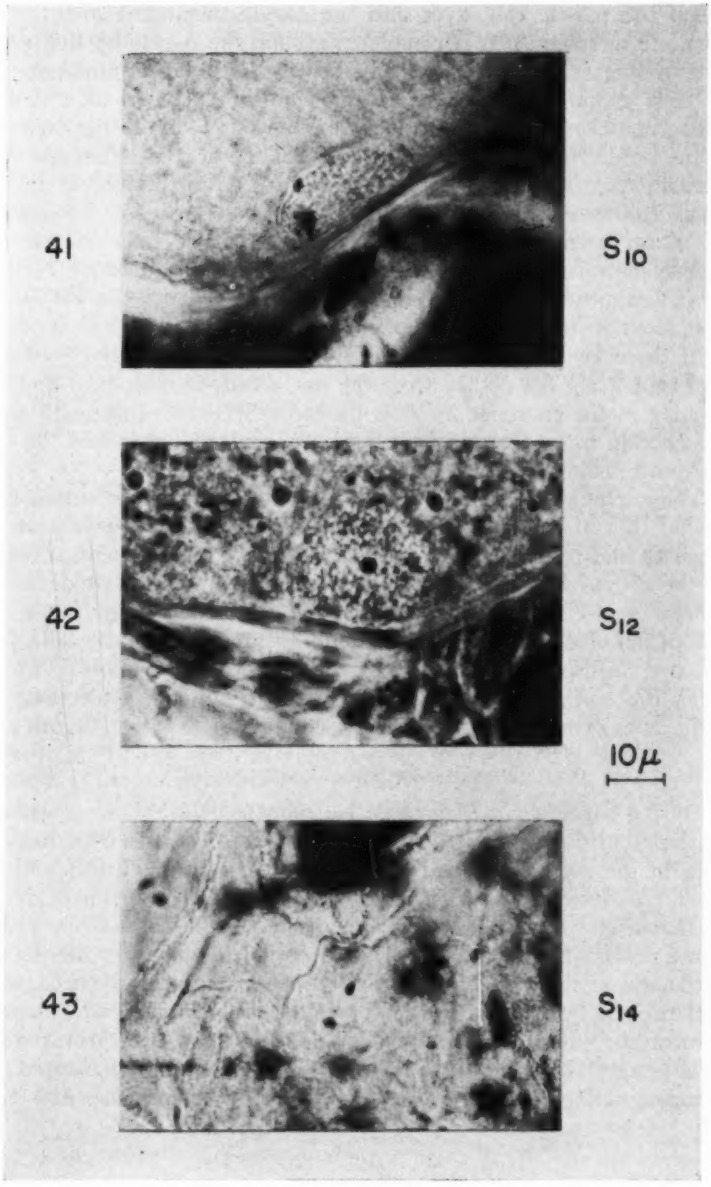

S7
33
S7
34
S8
35
S8
36
S8
P
K
37
38
BC
39
BC
S9
40
Ch
S9
BC
S9
10μ

FIGURE 41
Stage 10 oocyte nucleus. Actually Figure 24 at higher magnification.

FIGURE 42
Stage 12 oocyte nucleus. Actually Figure 26 at higher magnification.

FIGURE 43
The karyosphere of a stage 14 oocyte (directly in center of the field).


41
S10
42
S12
10μ
43
S14

tween the follicle cell layer and the oocyte membrane and is first seen late in stage 9. It is completed around the oocyte by the beginning of stage 12 (Figure 47). The "border cells" are a group of 6 to 10 cells containing nuclei 6μ in diameter (Figures 38-40 and 49). These non-choriogenic cells appear in the center of the egg chamber at the border between the nurse cells and the oocyte. They are first seen at stage 9 and presumably migrate in from peripheral follicle cells. However, in spite of a diligent search this proposed migration has never been observed. Late in stage 10 these cells migrate anteriorly between the nurse cells, and by the middle of stage 11 they have disappeared. It is interesting to note that the chorion is not completed in the region occupied by the border cells, and it is not until these cells migrate into the nurse chamber that the epithelial wall separating the oocyte from the nurse cells is completed and the opening in the chorionic cover is plugged. Sections of stage 13 show the chorion to be thrown up in folds. When the egg is laid the epithelium remains in the ovariole and degenerates.

The micropyle (Figures 27 and 29) which is formed in an anterior, medial, dorsal pocket of epithelial cells begins its development at stage 12 and is completed by stage 13. In the anterior, dorsal region of the oocyte follicle cells elongate during stage 13 to produce two tubular projections which are lateral to the degenerating nurse nuclei. Chorionic material secreted into the lumen of each tube produces a transparent lattice work (Figures 29-32 and 46). Later, when the epithelium is sloughed off these chorionic projections remain as the characteristic dorsal appendages of the egg (Figure 28).

Discussion and Conclusions

During oogenesis in *Drosophila melanogaster* the volume of ooplasm increases in 3 days by 100,000 times because of the deposition of yolk in the oocyte. Proteid yolk originates from precursors emitted from the nurse cell nuclei (Hsu, '52, '53), and therefore this remarkable growth is due at least in part to synthetic activity of the nurse nuclei. In preparation for this period of intense synthesis the chromatin of the nurse cells increases in mass and surface area. Hsu and Hansen ('53) have shown that nurse cell chromosomes increase in breadth, but to a greater extent in length. In the earliest nurse cells (stages 1 and 2) the homologous chromosomes are synapsed and attached at their centromere regions to a single nucleolus. At a time

which probably corresponds to stage 3 the chromosomes become thicker because each duplicates and splits into two chromatids. These paired chromosomes next lengthen and coil about one another, but they remain attached at their ends and so produce the bulbous condition characteristic of stage 4. Subsequently (stages 5-9) the chromosomes separate, elongate and spread out in the nucleus, and finally as we have shown the point is reached (stage 10) where lampbrush-type loops are observed which appear to originate from a chromosomal axis.

It is obvious that the DNA content per nurse cell nucleus increases during the developmental period which includes stages 6, 7, 8 and 9. Hsu and Hansen claim that the chromosomes do not increase in number during this period. In so doing these authors are in disagreement with Painter and Reindorp ('39). If Hsu and Hansen are correct then each chromosome must contain more DNA.

There are recorded in the literature many cases of endopolyploidy where an increase in chromosomal DNA is accompanied by longitudinal division of the chromosomes but not by subsequent cell division. There is also the converse situation where a chromosome increases in length (presumably by unfolding) without increasing in DNA content. There is no evidence, however, that a chromosome can synthesize DNA along its length and so grow longer. An objection may be raised in regard to the last generalization in view of the statement by Breuer and Pavan ('55) that the DNA content of certain bands of the chromosomes of *Rhynchosciara angelae* increases to an amount several times greater than that which the band had before undergoing bulb formation. However, photometric studies by Rudkin ('55) indicate in *Drosophila melanogaster* that puff or bulb formation is not attended by a change in nucleic acid content.

Therefore we are inclined to believe that if the chromosome number remains constant, then during stages 6, 7, 8 and 9 the chromosomes must become polytene as they elongate. As a result their girth does not change markedly. In this regard it is interesting to recall that Stalker ('54) has shown banded polytene chromosomes in the ovarian nurse cells of adults belonging to 16 species distributed among 7 families of Diptera. It is obvious that in the case of *Drosophila melanogaster* the "polytene" chromosomes of the nurse cell nuclei fail to show banding, a phenomenon so characteristic of the typical, polytene, salivary gland chromosomes.

FIGURE 44

A nucleus of a stage 10 nurse cell. Note the lateral loops extending from the chromosome axis.

FIGURE 45

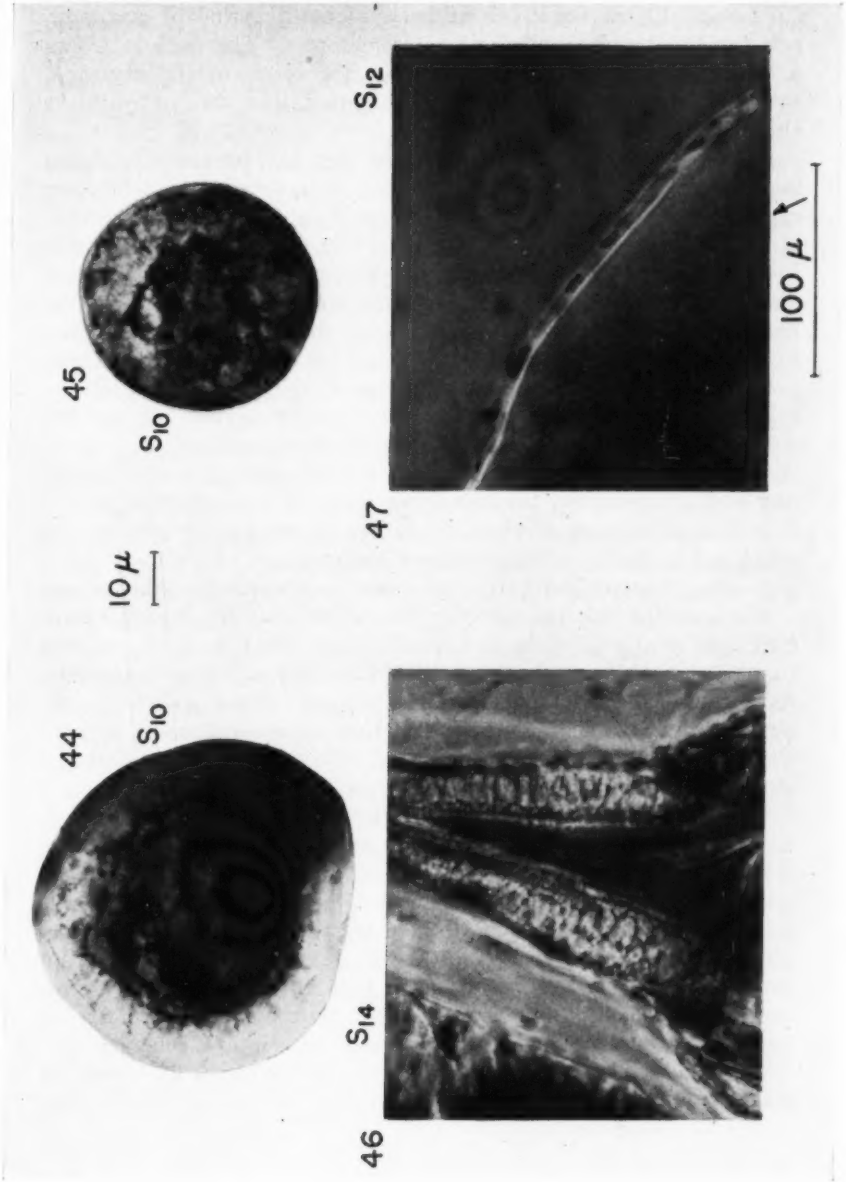
A nucleus of a stage 10 nurse cell showing the centrally arranged plasmosomes.

FIGURE 46

The basal region of the dorsal appendages showing the chorionic lattice work. Actually Figure 28 at a higher magnification.

FIGURE 47

A high power view of a stage 12 oocyte showing the chorion as it is laid down between the epithelial layer and the oocyte membrane.


45
S10
10 μ
44
S10
S12
47
100 μ
S14
46

At stage 10 the nurse cell nucleus reaches a period of maximum activity and becomes filled with plasmosomes. The nucleus shows a reticulate appearance which may be the result of the distorted, stretched, polytene chromosomes separating into their constituent chromatids.

King and Wilson ('55) demonstrated that 36% of the phosphorus ingested daily by the adult female fruit fly is incorporated into the eggs laid that same day. At the onset of yolk deposition the follicle cells above the nurse chamber differentiate into a squamous epithelium. Presumably nutrients from ingested yeast pass into the hemolymph and from there through the squamous epithelium surrounding the nurse cell chamber and into the nurse cell cytoplasm and nucleoplasm. Here these metabolites serve as raw materials for synthetic processes controlled by the chromosomes of the nurse cells. The resulting "yolk" is poured into the oocyte at the border between the oocyte and the nurse cells. At this region where synthesis and transport are most rapid the nurse cell nuclei are largest. During stages 9 and 10 the passageway through which material is entering the oocyte is in danger of being blocked by chorion secreted by epithelial cells which are gradually walling off the nurse chamber (Figure 40). Perhaps the border cells function somehow to prevent this obstruction.

Much of the material entering the oocyte may be related to nucleic acid synthesis, since Levenbook et al. ('53) have shown that the ooplasm of mature eggs is filled with polynucleotide fragments. As Painter ('40) has suggested, this material probably is resynthesized into nucleic acids during the period of intense mitotic activity which follows fertilization of the egg. Yao ('49) reports that the nurse cells of chambers at stages which probably correspond to 8 through 10 contain a high concentration of RNA in the cytoplasm, and that this RNA decreases during stages 11 and 12. Yao notes that a decrease in cytoplasmic RNA does not occur in the follicle cells, and he correlates this behavior with the fact that these cells are still synthetically active at these late stages, since they are responsible for the production of the chorion and the chorionic appendages. It is interesting to recall that at stage 7 (prior to the deposition of yolk in the oocyte) the egg chamber elongates, and the follicle cells above the oocyte differentiate into a columnar epithelium. The secretion of chorion occurs during stages 9-13, and the decrease in height of the columnar layer of follicle cells occurs dur-

ing stages 11-13. Whether these cells contribute any material to the egg other than chorion is unknown.

Stage 8 would appear to be a point of crisis in the development of the egg, since in normal females egg chambers often die and degenerate while in this stage (Figure 48). 1 day old females normally show 15% (27/177) pycnotic stage 8 chambers. Stages subsequent to 7 appear to be passed through relatively rapidly, since the ovaries of mature females killed during their period of maximum egg production show stages 1 through 7 and 14, but no intervening stages. The ovaries of 24-25 hour old females show all stages (except 14), but 11 is the rarest, suggesting that this stage is the most rapid of all.

In some insect species the nurse nuclei pass into the ooplasm (Peacock and Gresson, '28 and Kaufman, et al., '53). Occasionally one sees in *Drosophila* chambers in which it appears that nurse cell nuclei are engulfed by the oocyte (Figure 49). Actually these nuclei are within a finger-like projection of nurse cell cytoplasm and are therefore not completely cut off from the nurse cell chamber. Eventually these nuclei will be pushed by the expanding oocyte back into the nurse chamber. If nurse nuclei are absorbed into the ooplasm, it is a rare event, since counts made of the number of nurse nuclei in the anterior cap of fifty stage 12 chambers showed that there were always 15.

During this study we have observed many hundreds of ovarioles, and in only one case have we noted abnormalities in their development. The ovariole in question we found in one ovary of a 21 day old fly (mated from day 4 on); the other ovary was morphologically normal. The abnormal ovariole (Figures 50 and 51) contained a germarium (24 x 45μ), a germarial cyst (24 x 29μ) and two egg chambers (48 x 48 and 40 x 60μ). The germarial cyst was abnormally large and contained about 20 cells (each 8μ in diameter). Some of these cells were in various stages of division, and several multipolar mitoses were observed. Three cells contained nuclei which appeared empty except for characteristic karyosomes. The chambers each contained about 500 small cells[3] (6-7 μd), some of which were in active mitosis. A few giant cells in each chamber had diameters of 18μ. These cells appeared to be devoid of chromatin. Many cells in anaphase showed chromosome fragments. In the distal egg cham-

[3] Calculated from the formula $N = 0.7404\ (d_2/d_1)^3$, where N is the number of spheres of diameter d_1 which fit into a spherical space of diameter d_2.

FIGURE 48

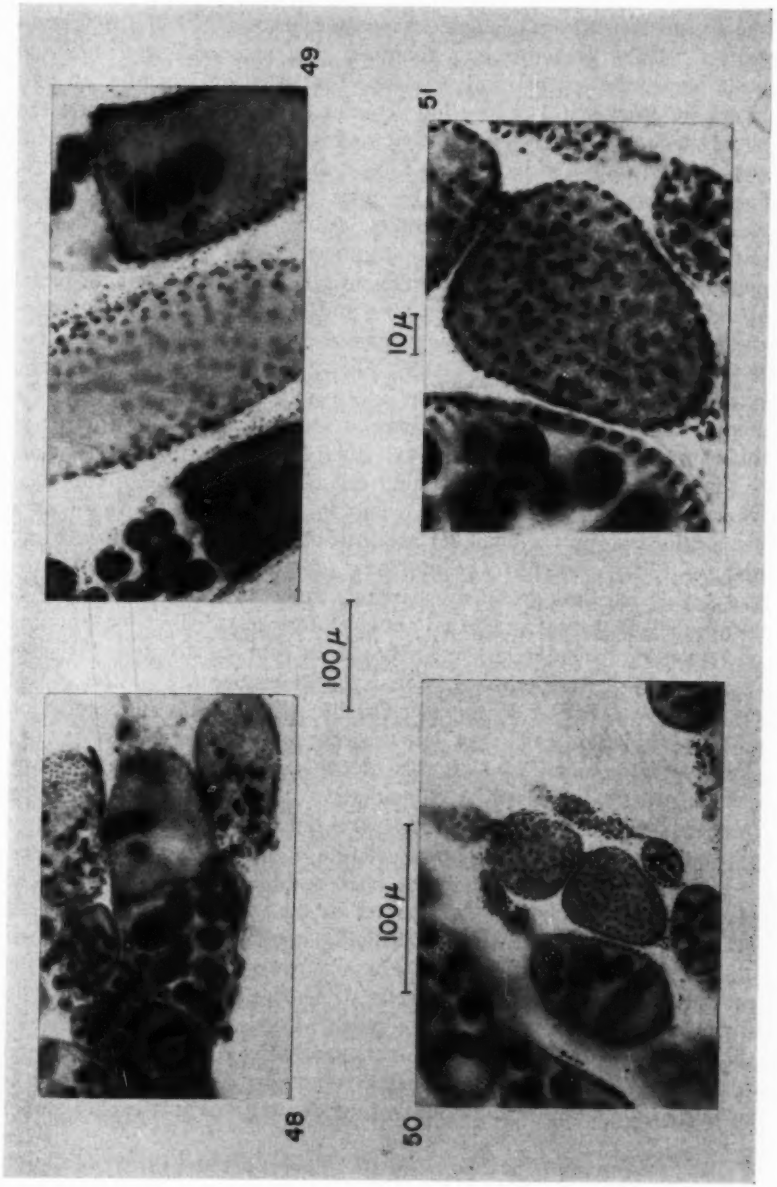
A whole mount of a group of ovarioles showing two degenerating, stage 8 chambers.

FIGURE 49

Stage 10 egg chambers of *D. virilis*. The size difference between nurse cell nuclei near the oocyte and those more anterior is striking. Note the cluster of border cells. In the right chamber nurse nuclei appear to be engulfed by the oocyte.

FIGURES 50 AND 51

Views at two magnifications of abnormal egg chambers found in a 21 day old fly.


49
51
10μ
100μ
48
50
100μ

ber six nuclei showed a single karyosome; three showed two karyosomes. In the posterior egg chamber nine nuclei showed one and three nuclei showed two karyosomes. The situation is similar to that found by Bucher ('51) in adult females irradiated early during the pupal stage. We have observed that such tumors appear regularly 1-2 weeks following irradiation (3-5 kr) of 2 hr. old adult females. Such abnormal chambers may be more frequent in older flies, and we shall look into this possibility further. At any rate, these chambers are clearly the result of abnormalities in the division of oogonial cells (possibly of common origin). Instead of an oogonial cell dividing four consecutive times and then ceasing, mitoses continued and were still in process when the fly was killed. It is obvious that dividing cells have lost the ability to differentiate into nurses, but can undergo differentiation into oocyte-like cells with karyosomes. Perhaps at the fourth division one of the 16 daughters was determined as a "pro-oocyte" and this cell, upon further division, produced the karyosome-containing cell observed. Possibly the position of cells rather than their origin is important, and those at a certain location in the chamber at a certain time are transformed into oocytes. These are but two of many possible speculations. At any event, this peculiar group of chambers gives us some interesting information. It appears (1) that those factors responsible for stopping an oogonium from undergoing more than four consecutive divisions sometimes fail to operate, (2) that the determination of the oocyte occurs before that of the nurse cells, (3) that nuclear differentiation of potential nurse cells may fail to occur under rare conditions, (4) that the frequency at which this morphogenetic abnormality occurs can be greatly increased by x-irradiation of females and (5) that a follicle layer develops about a cyst and pinches off from the germarium whether or not the enclosed cells have differentiated into nurses.

It has been indicated that genetic recombination has already taken place in chromosomes of the oocytes in the most anterior chambers. (Plough, '17), and consequently it is assumed that the stages of meiotic prophase which precede crossing over occur mainly in the germarium in nests of sixteen cells which are not as yet completely surrounded by a layer of follicle cells. Mature eggs in the ovarioles show a karyosphere lying free in the ooplasm at the base of the dorsal appendages. The karyosphere has a volume of about 2.7 $\mu 3$, which is sufficient to contain tetrads of the four chromosomes, if each member

of the tetrad has the dimensions of a corresponding chromosome as viewed in an oogonial metaphase. The oocytes of almost all egg chambers show the karyosome lying in a reticulate nucleoplasm which is surrounded by a nuclear membrane. Since diakinesis (Fahmy, '52) and metaphase I and II (Fahmy, '52 and Sonnenblick, '50) occur subsequent to the time the egg leaves the ovariole, the karyosome may represent a modified diplotene stage. At any rate karyosome stages include cells which have undergone crossing over, but which have not reached diakinesis. Feulgen-positive nucleoli are present in the nuclei of other tissues in *Drosophila melanogaster*. These tissues include: (1) the larval salivary glands, (2) the larval fat body cells (Figures 54 and 55) and, (3) the cells of the columnar epithelium of egg chambers (Figure 53). Such karyosomes probably represent an association of the heterochromatic elements of the various chromosomes. In the case of the salivary gland chromosomes the karyosome is called the chromocenter. The karyosome of the oocyte nucleus may be a similar aggregation of heterochromatic elements. In early stages chromosome strands are associated with the karyosome. However, most later stages have nuclei in which the karyosome lies in a reticulate nucleoplasm, and it is impossible (at least in our preparations) to decide whether or not this reticulate appearance is due to chromosomes (attached to the karyosome) that are so thin and weakly staining as to be hardly visible. If such chromosomes do exist, they condense and are incorporated into the karyosphere characteristic of stage 14 eggs. Alternatively, the karyosome in nuclei of oocytes in stages 9-13 may represent an aggregation of all the chromatin of the nucleus, not just the heterochromatin. Swift and Kleinfeld ('53) have shown that throughout development the primary oocytes of *Melanoplus differentialis* contain the 4c amount of DNA, and we assume a similar situation exists in *Drosophila melanogaster*.

Summary

The development of the egg in the ovariole of *Drosophila melanogaster* has been divided into 14 successive stages. Differences are described between the proportions of the chambers in various stages and the total numbers of chambers found in females of various ages. Descriptions are given of the development of the egg chamber, the oocyte, the nurse cells, and of the follicle cells and associated struc-

FIGURE 52

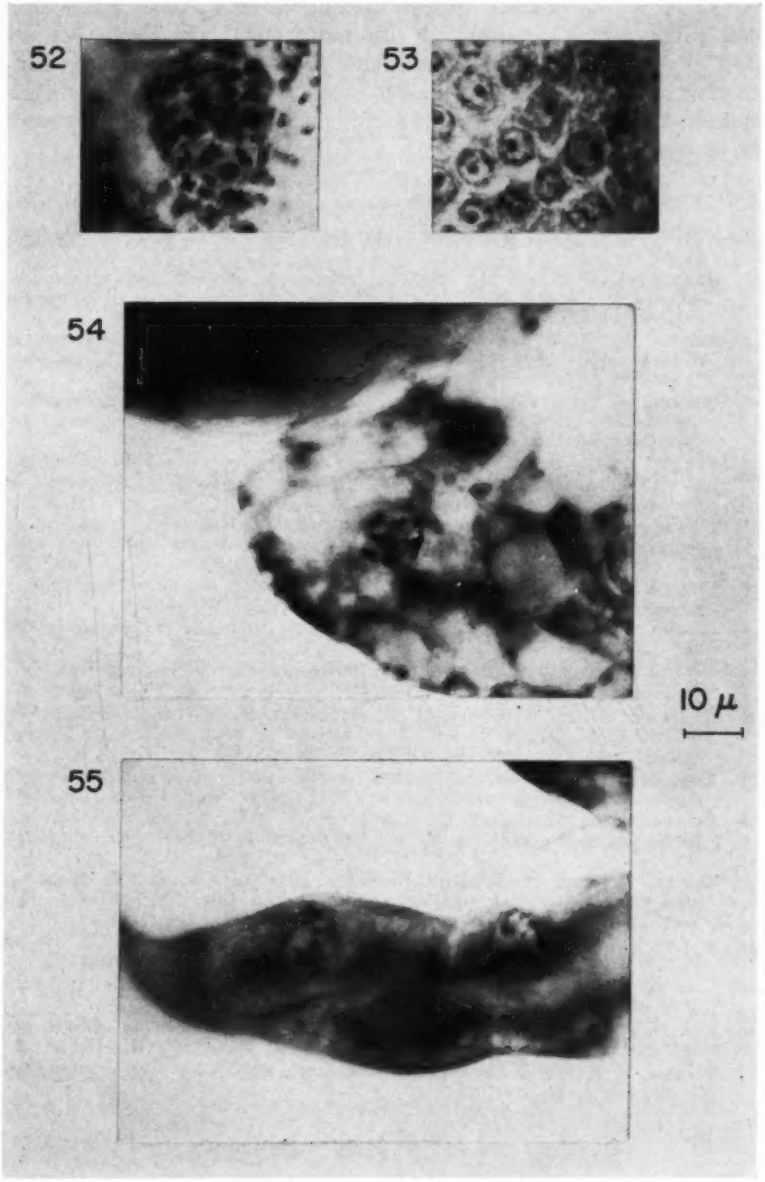
Synchronous divisions in the germarium. Six of eight anaphases are shown. The resulting 16 cells will form a germarial cyst.

FIGURE 53

Nuclei of the cells of the columnar epithelium of stage 10 oocytes. Feulgen-positive karyosomes are clearly visible.

FIGURES 54 AND 55

Nuclei of fat body cells of third instar *Drosophila melanogaster* larvae. Note the karyosomes to which the chromosomes are attached.


52
53
54
10 μ
55

tures. The relationship between the nurse cells, their nuclei and yolk production is discussed. A rare morphologic abnormality occurring in one ovariole is described, and the information it gives concerning the production of the normal egg chamber is discussed. Conclusions are drawn as to the condition of the oocyte chromosomes at various stages of oogenesis.

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PREPARATIONS

1. Freeze dehydrated whole mounts, unstained, phase contrast. Figures 3, 27.
2. Baker's fixative, Feulgen stained whole mounts, phase contrast. Figures 4-6.
3. Kahle's fixative, Feulgen stained whole mounts, phase contrast. Figures 7-12, 14-16, 19-21, 33-42, 45, 46.
4. Aqueous Bouin's fixative, toluidine blue sections, bright field. Figure 30.
5. Aqueous Bouin's fixative, iron haematoxylin sections, bright field. Figures 29, 31, 32.
6. Kahle's fixative, Feulgen stained whole mounts, bright field. Figures 13, 17, 18, 22-26, 28, 43, 44, 48-55.
7. Kahle's fixative, Feulgen stained whole mounts, polarized light. Figure 47.

ORIENTATION

In Figures 3-32 the convention followed whenever possible is to have the anterior end at the left and the dorsal side uppermost.

ABBREVIATIONS

BC	border cells
C_1	incipient cyst
CH	chorion
K	karyosome
P	plasmosome
S	stage

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